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Ase Damm

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PATENT- OG VAREMÆRKESTYRELSEN

FLUOROPHORE COMPLEMENTATION PRODUCTS

Field of invention

The present invention relates to various split fluorophore complementation products, especially ways to obtain intense systems with GFP.

Background of the invention

It has been suggested to use the reassembly of certain enzyme fragments of the complete enzyme as a measure of protein-protein interactions. Johnsson and Varshavsky (Johnsson, N., Varshavsky, A. (1994) Proc. Natl. Acad. Sci. U. S. A. **91**, 10340-10344) disclose reassembly of Ubiquitin. This reassembly is detected through the irreversible cleavage of the fusion by Ubiquitin protease and release of a reporter. As opposed to the two-hybrid technique, this technique includes the possibility of monitoring a protein-protein interaction as a function of time, at the natural sites of this interaction in a living cell.

Similar systems are suggested for the reassembly of other proteins including β-galactosidase (Rossi, F., Charlton, C.A., Blau, H.M. (1997) Proc. Natl. Acad. Sci. U. S. A. **94**, 8405-8410), dihydrofolate reductase (DHFR, WO98/34120), and β-lactamase (Wehrman, T., Kleaveland, B., Her, J.H., Balint, R.F., Blau, H.M. (2002) Proc. Natl. Acad. Sci. U. S. A. **99**, 3469-3474). The basic concept is that by splitting a functional protein in two fragments, the function is lost. The two fragments are transformed or transfected into cells fused in frame to proteins X and Y, respectively. Binding between proteins X and Y will bring the two fragments close together, increasing the local concentration of the complementing fragments, induce folding of these fragments and produce a functional protein with an activity that is similar to that of the non-fragmented protein. If the function is DHFR activity, the cells will survive only if proteins X and Y bind to each other.

Recently, it has been described to use a somewhat similar system for the assisted reassembly and folding of fragments of fluorescent proteins. As the function is fluorescence, the cells will emit light upon excitation only if protein X and protein Y bind to each other thereby assisting complementation.

Ghosh (I. Ghosh, A.D. Hamilton, L. Regan (2000) J. Am. Chem. Soc. **122**, 5658-5659, WO01/87919) describes the use of a GFP variant called sg100 (F64L, S65C, Q80R, Y151L, I167T and K238N). This GFP has single fluorescence excitation and emission peaks at 475 nm and 505 nm, respectively (similar to sg25 described by Palm (Palm, G.J., Zdanov, A., Gaitanaris, G.A., Stauber, R., Pavlakis, G.N., Wlodawer, A. (1997) Nat. Struct. Biol. **4**, 361-365)).

Functional GFP fragment complementation is accomplished by co-expressing two independent peptides composed of the first 157 N-terminal amino acids of this GFP (NGFP) and the remaining 81 C-terminal amino acids (starting form residue 158) of this GFP (CGFP) with each of the GFP peptide fragments being fused to interacting leucine zipper peptides that serve to associate the fragments.

Nagai (T. Nagai, A. Sawano, E.S. Park, A. Miyawaki (2001) Proc. Natl. Acad. U. S. A. **98**, 3197-3202) tests a yellow fluorescent GFP variant that has the following mutations: S65G, V68L, Q69K, S72A, T203Y. This variant was split between residues N144 and Y145 within the open 129-145 loop region, and the peptides fused to M13 and calmodulin, respectively, for use in a Ca^{2+} assay. However, when the constructs were transfected individually into HeLa cells, the assay was not reliable.

Hu (Chang-Deng Hu, Yurii Chinenov, and Tom Kerppola (2002) Mol. Cell **9**, 789-798) enables the use of the GFP variant called EYFP (S65G, S72A, T203Y). The two GFP fragments which are described to work are the first 154 amino acids of GFP in one construct and amino acids 155 through 238 in the other construct.

However, there is still a need for alternative GFP's for use in this technology.

Summary of the invention

The present application discloses that certain GFPs can be reassembled and form a functional fluorescent protein when expressed as two independent proteins. For example, when EGFP is expressed in mammalian cells, choosing a split site located in a loop region between the residues that form the beta-sheet structures of the GFP beta-barrel results in intense fluorescence (Example 5 and Example 7). The present application further illustrates that EYFP is also reassembled and, surprisingly, the fluorescence from

the reassembled protein is markedly enhanced if it contains the F64L mutation (Example 9).

Detailed disclosure

The non-fluorescent fragments of fluorescent proteins that can be combined to form one functional fluorescent unit are usually produced by splitting the coding nucleotide sequence of one fluorescent protein at an appropriate site and expressing each nucleotide sequence fragment independently. The fluorescent protein fragments may be expressed alone or in fusion with one or more protein fusion partners.

Thus, one aspect of the invention relates to two GFP fragments comprising an N-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number 1 to amino acid number X of GFP, wherein the peptide bond between amino acid number X and amino acid number X+1 is within a loop of GFP, the two GFP fragments also comprise a C-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number X+1 to amino acid number 238 of GFP.

Amino acid 1 is meant to indicate the first amino acid of GFP. Amino acid 238 is meant to indicate the last amino acid of the GFP.

All residues are numbered according to the numbering of wild type A. victoria GFP (GenBank accession no. M62653) and said numbering also applies to equivalent positions in homologous sequences exemplified by alignment of fluorescent protein sequences in Example 1. Thus, when working with truncated GFPs (compared to wild type GFP) or when working with GFPs with additional amino acids, the numbering is relative to the alignment.

Green Fluorescent Protein (GFP) is a 238 amino acid long protein derived from the jellyfish *Aequorea Victoria* (SEQ ID NO: 1). However, fluorescent proteins have also been isolated from other members of the Coelenterata, such as the red fluorescent protein from *Discosoma* sp. (Matz, M.V. *et al.* 1999, Nature Biotechnology 17: 969-973), GFP from *Renilla reniformis*, GFP from *Renilla Muelleri* or fluorescent proteins from other animals, fungi or plants. The GFP exists in various modified forms including the blue fluorescent variant of GFP (BFP) disclosed by Heim et al. (Heim, R. *et al.*, 1994, Proc.Natl.Acad.Sci. 91:26, pp 12501-12504) which is a Y66H variant of wild type GFP; the yellow fluorescent

variant of GFP (YFP) with the S65G, S72A, and T203Y mutations (WO98/06737); the cyan fluorescent variant of GFP (CFP) with the Y66W colour mutation and optionally the F64L, S65T, N146I, M153T, V163A folding/solubility mutations (Heim, R., Tsien, R.Y. (1996) Curr. Biol. 6, 178-182). The most widely used variant of GFP is EGFP with the F64L and S65T mutations (WO 97/11094 and WO96/23810) and insertion of one valine residue after the first Met. The F64L mutation is the amino acid in position 1 upstream from the chromophore. GFP containing this folding mutation provides an increase in fluorescence intensity when the GFP is expressed in cells at a temperature above about 30°C.

It is known that fluorescence in wild-type GFP is due to the presence of a chromophore, which is generated by cyclisation and oxidation of the SYG at position 65-67 in the predicted primary amino acid sequence and presumably by the same reasoning of the SHG sequence and other GFP analogues at positions 65-67.

The present examples clearly illustrate how the fluorescence intensity from a reassembled protein is enhanced in GFPs containing the F64L mutations as compared to GFPs without this mutation. Thus, it is preferred that the GFP contains the F64L mutation, either by electing a GFP with this mutation (e.g. EGFP) or to introduce this mutation into the GFP of choice (e.g. YFP as illustrated in Example 8).

In the nomenclature of GFP, an "E" is placed in front of the GFP (EGFP, EYFP, ECFP) to indicate that this particular GFP is encoded by a nucleic acid with codon usage optimised for mammalian cells. Most of these proteins also have an extra valine residue inserted after the initial methionine residue, Met^1. This residue is not considered in the numbering of the residues. Thus, in a preferred embodiment, the GFP of the present invention is selected from the group consisting of EGFP, EYFP, ECFP, dsRed and Renilla GFP.

Some of the examples of the present application, EGFP is used. Thus, in a preferred embodiment of the invention, the GFP is EGFP. However, Example 8 and Example 9 show that EYFP has certain advantages. Thus, in another preferred embodiment of the invention, the GFP is EYFP.

In the present context, the numbering of wild-type GFP (SEQ ID NO: 1) (Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C. (1994) Science **263**, 802-805, this variant of GFP has a histidine residue in position 231) is used. Based on the crystal structure of GFP (Yang, F., Moss, L.G., Phillips, G.N. (1996) Nat. Biotech. **14**, 1246-1251) Figure 5,

Table 1 and the data presented in the examples, it is evident that a split in almost any loop will be re-assembled following appropriate spatial approximation to the complementation fragments assisted by the interaction of the conjugated proteins. For the purpose of this application the term "loop" shall be understood as a turn or element of irregular secondary structure.

Thus, in one aspect, the invention relates to two GFP fragments as described above, wherein X is 7, 8, 11 or 12, preferably X is 9 or 10 within the Thr9-Val11 loop; or wherein X is 21, 22, 25 or 26, preferably X is 23 or 24 within the Asn23-His25 loop; or wherein X is 36, 37, 40 or 41, preferably X is 38 or 39 within the Thr38-Gly40 loop; or wherein X is 46, 47, 56 or 57, preferably X is between 48 and 55 i.e. X is 48, 49, 50, 51, 52, 53, 54 or 55 within the Cys48-Pro56 loop; or wherein X is 70, 71, 76 or 77, preferably X is between 72 and 75 i.e. X is 72, 73, 74 or 75 within the Ser72-Asp76 loop; or wherein X is 79, 80, 83 or 84, preferably X is 81 or 82 within the His81-Phe83 loop; or wherein X is 86, 87, 90 or 91, preferably X is 88 or 89 within the Met88-Glu90 loop; or wherein X is 99, 100, 103 or 104, preferably X is 101 or 102 within the Lys101-Asp103 loop; or wherein X is 112, 113, 118 or 119, preferably X is between 114 and 117 i.e. X is 114, 115, 116 or 117 within the Phe114-Thr118 loop; or wherein X is 126, 127, 145 or 146, preferably X is between 128 and 144 i.e. X is 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144 within the Ile 128-Tyr145 loop; or wherein X is 152, 153, 160 or 161, preferably X is between 154 and 159 i.e. X is 154, 155, 156, 157, 158 or 159 within the Ala154-Gly160 loop; or wherein X is 169, 170, 175, 176, preferably X is between 171 and 174 i.e. X is 171, 172, 173 or 174 within the Ile171-Ser175 loop; or wherein X is 186, 187, 197 or 198, preferably X is between 188 and 196 i.e. X is 188, 189, 190, 191, 192, 193, 194, 195 or 196 within the Ile188-Asp197 loop; or wherein X is 208, 209, 215 or 216, preferably X is between 210 and 214 i.e. X is 210, 211, 212, 213 or 214 within the Asp210-Art215 loop.

Table 1 GFP secondary structures, GFP wild type sequence amino acid numbering. α and β indicate α-helical and β-sheet secondary structures, respectively.

Name	Position	
Helix 1	Lys3 - Thr9	α1
Sheet 1	Val11 - Asn23	β1
Sheet 2	His25 - Thr38	β2
Sheet 3	Gly40 - Cys48	β3
Helix 2	Pro56 - Ser72	α2
Helix 3	Asp76 - His81	α3

Helix 4	Phe83 - Met88	α4
Sheet 4	Glu90- Lys 101	β4
Sheet 5	Asp103 -Phe114	β5
Sheet 6	Thr118 - Ile128	β6
Sheet 7	Tyr145 - Ala154	β7
Sheet 8	Gly160 - Ile171	β8
Sheet 9	Ser175 - Ile188	β9
Sheet 10	Asp197 - Asp210	β10
Sheet 11	Arg215 - Gly228	β11

Based on the findings disclosed in the examples, it is concluded that appropriate splitting sites in GFP are located in the loop regions between the residues that form the beta-sheet structures of the GFP beta-barrel. Accordingly, splits in GFP are preferably made in the Asn23-His25 loop, the Thr38-Gly40 loop, the Lys101-Asp102 loop, the Phe114-Thr118 loop, the Ile128-Tyr145 loop, the Ala154-Gly160 loop, the Ile171-Ser175 loop, the Ile188-Asp197 loop or the Asp210-Arg215 loop (Table 1, Figure 5).

The data in the present examples illustrates clearly that the Ala154-Gly160 loop is very well suited for GFP reassembly. This is particularly the case when the GFP is divided between amino acids Q157 and K158 (that is, when X is 157). Thus, a preferred embodiment of the invention relates to two GFP fragments, wherein X is 157 within the Ala154-Gly160 loop.

The data in the present examples also illustrate that the Ile171-Ser175 loop is very useful for GFP reassembly. This is particularly the case, when the GFP is divided between amino acids E172 and D173 (that is, when X is 172). Thus, a preferred embodiment of the invention relates to two GFP fragments, wherein X is 172 within the Ile171-Ser175 loop.

As illustrated in Example 9, fragments having overlapping sequences are also functional. Thus one aspect of the invention relates to two GFP fragments comprising
(a) an N-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number 1 to amino acid number X of GFP, wherein the peptide bond between amino acid number X and amino acid number X+1 is within a loop of GFP and
(b) a C-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number Y to amino acid number 238 of GFP, wherein Y<X creating an overlap

of the two GFP fragments, and wherein the peptide bond between amino acid Y-1 and amino acid Y is within a loop of GFP.

These overlapping GFP fragments are very attractive in e.g. functional cloning systems where highly flexible linkers sequences are required due to the very diverse nature of the fusion partners. The overlapping fragments permit either of the fusion partners to have a long linker sequence.

For the purposes of deciding the nature of the Y in the C-terminal fragment of GFP defined above, the same considerations as discussed for the value of X applies.

In order to obtain reassembly of the two halves of GFP, it is essential to have the two halves of GFP fused to interaction partners that will bring said two halves of GFP so close together that the protein halves will fold and form functional GFP (see Hu 2002 supra). Thus, a preferred embodiment of the invention relates to a fusion protein comprising an N-terminal fragment of GFP as described above conjugated to a first protein of interest. In a particular embodiment the N-terminal fragment of GFP is fused in frame to the first protein of interest. In similar embodiments, the present invention relates to two GFP fragments as described above, wherein the C-terminal fragment of GFP is conjugated to a second protein of interest. In a particular embodiment, the C-terminal fragment of GFP is fused in frame to the second protein of interest.

As will be evident to the skilled person, the protein of interest is fused to the GFP fragment in the N-terminal or in the C-terminal. However, as illustrated in the examples, fusion of the first protein of interest to the N-terminal fragment of GFP shall preferably be to the C-terminal of the N-terminal fragment of GFP. Likewise, fusion of the second protein of interest to the C-terminal fragment of GFP shall preferably be to the N-terminal of the C-terminal fragment of GFP.

As will be evident from the present examples, and e.g. Hu (2002 supra), the protein of interest is a protein, a peptide or a non-proteinaceous partner.

In a typical embodiment of the invention, the fusion protein as described above, wherein the fragment of GFP is fused in frame to a protein of interest, further comprises a linker sequence between either fragment of GFP and the corresponding protein of interest.

The linker must be chosen dependent on the protein of interest conjugated to the fragment of GFP. Thus the linker must be flexible. A long linker prevent steric hindrance of the complementation due to the protein of interest. However short linkers keeps the fragments of GFP closer to each other and gives better associations.

The present invention also relates to the N-terminal fragment of GFP as described above. In a similar embodiment, the invention relates to the C-terminal fragment of GFP as described above.

A preferred embodiment of the invention relates to a Nucleic acid encoding any of the fragments or fusions proteins described above. In one embodiment, the nucleic acid construct encoding any of the proteins according to the invention described above is a DNA construct. In another embodiment, the nucleic acid construct encoding any of the proteins according to the invention described above is a RNA construct.

One aspect of the invention relates to a cell containing the two GFP fragments described above. In similar embodiments, the invention relates to a cell containing the N-terminal fragment of GFP described above. In similar embodiments, the invention relates to a cell containing the C-terminal fragment of GFP described above.

Numerous cell systems for transfection exist. A few examples of mammalian cells isolated directly from tissues or organs taken from healthy or diseased animals (primary cells), or transformed mammalian cells capable of indefinite replication under cell culture conditions (cell lines). The term "mammalian cell" is intended to indicate any living cell of mammalian origin. The cell may be an established cell line, many of which are available from The American Type Culture Collection (ATCC, Virginia, USA) or similar Cell Culture Collections. The cell may be a primary cell with a limited life span derived from a mammalian tissue, including tissues derived from a transgenic animal, or a newly established immortal cell line derived from a mammalian tissue including transgenic tissues, or a hybrid cell or cell line derived by fusing different cell types of mammalian origin e.g. hybridoma cell lines. The cells may optionally express one or more non-native gene products, e.g. receptors, enzymes, enzyme substrates, prior to or in addition to the fluorescent probe. Preferred cell lines include, but are not limited to, those of fibroblast origin, e.g. BHK, CHO, BALB, NIH-3T3 or of endothelial origin, e.g. HUVEC, BAE (bovine artery endothelial), CPAE (cow pulmonary artery endothelial), HLMVEC (human lung micro vascular endothelial cells), or of airway epithelial origin, e.g. BEAS-2B, or of

pancreatic origin, e.g. RIN, INS-1, MIN6, bTC3, aTC6, bTC6, HIT, or of hematopoietic origin, e.g. primary isolated human monocytes, macrophages, neutrophils, basophils, eosinophils and lymphocyte populations, AML-14, AML-193, HL-60, RBL-1, U937, RAW, JAWS, or of adipocyte origin, e.g. 3T3-L1, human pre-adipocytes, or of neuroendocrine origin, e.g. AtT20, PC12, GH3, muscle origin, e.g. SKMC, A10, C2C12, renal origin, e.g. HEK 293, LLC-PK1, or of neuronal origin, e.g. SK-N-DZ, SK-N-BE(2), HCN-1A, NT2/D1.

One aspect of the invention relates to a method for detecting the interaction between two proteins of interest comprising the steps of:

(a) providing at least one cell that contains two heterologous conjugates,
the first heterologous conjugate comprising a first protein of interest conjugated to an N-terminal fragment of GFP as described above,
the second heterologous conjugate comprising a second protein of interest conjugated to a C-terminal fragment of GFP as described above; and

(b) measuring the fluorescence from the at least one cell,

fluorescent cells indicating interaction between the two proteins of interest.

In a similar embodiment, the invention relates to a method for monitoring the interaction between two proteins of interest comprising the steps of:

(a) providing at least one cell containing at least one stretch of nucleic acid encoding two heterologous conjugates:
the first heterologous conjugate comprising a first protein of interest conjugated to an N-terminal fragment of GFP as described above,
the second heterologous conjugate comprising a second protein of interest conjugated to a C-terminal fragment of GFP as described above;

(b) culturing the at least one cell under conditions allowing expression; and

(c) measuring the fluorescence from the at least one cell,

fluorescent cells indicating interaction between the two proteins of interest.

In one aspect of the methods, one of the proteins of interest is known, whereas the other protein of interest is an unknown protein. By parallel transfection the cells with interaction partners to the know protein of interest will be fluorescent and thereby easily detectable.

In a preferred aspect of the methods, the at least one cell is a mammalian cell.

In another preferred aspect of the methods, the heterologous conjugates are fusion proteins.

This technology has broad applicability. Due to the direct detection of interactions is can be used in genomics and proteomics. The high sensitivity makes is applicable to target discovery and the high specificity makes it applicable to target validation. It can be scaled to Drug Discovery in High Throughput Screening. The technology is quantitative and makes it applicable to nanotechnology.

The invention will be illustrated more specifically in the following non-limiting examples.

Examples

Example 1: Alignment of fluorescent proteins

GenBank entry	Fluorescent protein
P42212	Aequorea victoria green-fluorescent protein
AF372525	Renilla reniformis green fluorescent protein
AY015996	Renilla muelleri green fluorescent protein
AY013824	Aequorea macrodactyla isolate GFPxm
AF384683	Montastraea cavernosa green fluorescent protein
AF401282	Montastraea faveolata green fluorescent protein
AY015995	Ptilosarcus sp. CSG-2001 green fluorescent protein
AF322221	Anemonia sulcata green fluorescent protein asFP499
AF322222	Anemonia sulcata nonfluorescent red protein asCP562
AF246709	Anemonia sulcata GFP-like chromoprotein FP595
AF168419	DsRed Discosoma sp. fluorescent protein FP583
AF168420	Discosoma striata fluorescent protein FP483
AF168421	Anemonia majano fluorescent protein FP486
AF168422	Zoanthus sp. fluorescent protein FP506
AF168423	Zoanthus sp. fluorescent protein FP538
AF168424	Clavularia sp. fluorescent protein FP484

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P42212     avGFP ----------------------------------------MSKGEELFTGVVPILVELDGDV 22
AY015996   rmGFP ----------------------------------MSKQILKNTCLQEVMSYKVNLEGIV 25
AF372525   rrGFP ------------------------------------MD---LAKLGLKEVMPTKINLEGLV 22
AF168419   dsRed ----------------------------------------MRSSKNVIKEFMRFKVRMEGTV 22
AF322222 asCP562 ----------------------------------------MASFLKKTMPFKTTIEGTV 19
AF168422 asCP562 ----------------------------------MAQSKHGLTKEMTMKYRMEGCV 22
AF246709 asFP595 ----------------------------------------MASFLKKTMPFKTTIEGTV 19
AF322221 asFP499 ----------------------------------------MYPSIKETMRVQLSMEGSV 19
AF384683   mcGFP ------------------------------------------MSVIKPIMEIKLRMQGVV 18
AF401282   mfGFP ------------------------------------------MSVIKPDMKIKLRMEGAV 18
AF168424 csFP484 MKCKFVFCLSFLVLAITNANIFLRNEADLEEKTLRIPKALTTMGVIKPDMKIKLKMEGNV 60
AF168420 dsFP483 ----------------------------------MSCSKSVIKEEMLIDLHLEGTF 22
AY015995   spGFP ----------------------------------MNRNVLKNTGLKEIMSAKASVEGIV 25
AF168423 zsFP538 ------------------------------------MAHSKHGLKEEMTMKYHMEGCV 22
AF168421 amFP486 ------------------------------------MALSNKFIGDDMKMTYHMDGCV 22
AY013824 amGFPxm ------------------------------------MSKGEELFTGIVPVLIELDGDV 22
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P42212     avGFP NGHKFSVSGEGEGDATYGKL--TLKFICTTG-KLPVPWPTLVTTFSYGVQCFSRYPDHMK 79
AY015996   rmGFP NNHVFTMEGCGKGNILFGNQ--LVQIRVTKGAPLPFAFDIVSPAFQYGNRTFTKYPNDIS 83
AF372525   rrGFP GDHAFSMEGVGEGNILEGTQ--EVKISVTKGAPLPFAFDIVSVAFSYGNRAYTGYPEEIS 80
AF168419   dsRED NGHEFEIEGEGEGRPYEGHN--TVKLKVTKGGPLPFAWDILSPQFQYGSKVYVKHPADIP 80
AF322222 asCP562 NGHYFKCTGKGEGNPFEGTQ--EMKIEVIEGGPLPFAFHILSTSCMYGSKTFIKYVSGIP 77
AF168422 asCP562 DGHKFVITGEGIGYPFKGKQ--AINLCVVEGGPLPFAEDILSAAFNYGNRVFTEYPQDIV 80
AF246709 asFP595 NGHYFKCTGKGEGNPFEGTQ--EMKIEVIEGGPLPFAFHILSTSCMYGSKTFIKYVSGIP 77
AF322221 asFP499 NYHAFKCTGKGEGKPYEGTQ--SLNITITEGGPLPFAFDILSHAFQYGIKVFAKYPKEIP 77
AF384683   mcGFP NGHKFVIKGEGEGKPFEGTQ--TINLTVKEGAPLPFAYDILTSAFQYGNRVFTKYPDDIP 76
AF401282   mfGFP NGHKFVIEGDGKGKPFEGTQ--SMDLTVKEGAPLPFAYDILTTVFDYGNRVFAKYPQDIP 76
AF168424 csFP484 NGHAFVIEGEGEGKPYDGTH--TLNLEVKEGAPLPFSYDILSNAFQYGNRALTKYPDDIA 118
AF168420 dsFP483 NGHYFEIKGKGKGQPNEGTN--TVTLEVTKGGPLPFGWHILCPQFQYGNKAFVHHPDNIH 80
AY015995   spGFP NNHVFSMEGFGKGNVLFGNQ--LMQIRVTKGGPLPFAFDIVSIAFQYGNRTFTKYPDDIA 83
AF168423 zsFP538 NGHKFVITGEGIGYPFKGKQ--TINLCVIEGGPLPFSEDILSAGFKYGDRIFTEYPQDIV 80
AF168421 amFP486 NGHYFTVKGEGNGKPYEGTQTSTFKVTMANGGPLAFSFDILSTVFKYGNRCFTAYPTSMP 82
AY013824 amGFPXM HGHKFSVRGEGEGDADYGKL--EIKFICTTG-KLPVPWPTLVTTFSYGIQCFARYPEHMK 79
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P42212      avGFP QHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEV--KFEG---DTLVNRIELKGIDFKEDG 134
AY015996    rmGFP --DYFIQSFPAGFMYERTLRYEDGGLVEIRSDI--NLIE---DKFVYRVEYKGSNFPDDG 136
AF372525    rrGFP --DYFLQSFPEGFTYERNIRYQDGGTAIVKSDI--SLED---GKFIVNVDFKAKDLRRMG 133
AF168419    dsRED --DYKKLSFPEGFKWERVMNFEDGGVVTVTQDS--SLQD---GCFIYKVKFIGVNFPSDG 133
AF322222 asCP562 --DYFKQSFPEGFTWERTTTYEDGGFLTAHQDT--SLDG---DCLVYKVKILGNNFPADG 130
AF168422 asCP562 --DYFKNSCPAGYTWDRSFLFEDGAVCICNADITVSVEEN---CMYHESKFYGVNFPADG 135
AF246709 asFP595 --DYFKQSFPEGFTWERTTTYEDGGFLTAHQDT--SLDG---DCLVYKVKILGNNFPADG 130
AF322221 asFP499 --DFFKQSLPGGFSWERVSTYEDGGVLSATQET--SLQG---DCIICKVKVLGTNFPANG 130
AF384683    mcGFP --DYFKQTFPEGYSWERIMAYEDQSICTATSDI--KMEG---DCFIYEIQFHGVNFPPNG 129
AF401282    mfGFP --DYFKQTFPEGYSWERSMTYEDQGICVATNDI--TLMKGVDDCFVYKIRFDGVNFPANG 132
AF168424 csFP484 --DYFKQSFPEGYSWERTMTFEDKGIVKVKSDI--SMEE---DSFIYEIRFDGMNFPPNG 171
AF168420 dsFP483 --DYLKLSFPEGYTWERSMHFEDGGLCCITNDI--SLTG---NCFYYDIKFTGLNFPPNG 133
AY015995    spGFP --DYFVQSFPAGFFYERNLRFEDGAIVDIRSDI--SLED---DKFHYKVEYRGNGFPSNG 136
AF168423 zsFP538 --DYFKNSCPAGYTWGRSFLFEDGAVCICNVDITVSVKEN---CIYHKSIFNGMNFPADG 135
AF168421 amFP486 --DYFKQAFPDGMSYERTFTYEDGGVATASWEI--SLKGN---CFEHKSTFHGVNFPADG 135
AY013824 amGFPXM MNDFFKSAMPEGYIQERTIFFQDDGKYKTRGEV--KFEG---DTLVNRIELKGMDFKEDG 134
          *: : * * * ::* . : . : . .: *

P42212      avGFP NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQN--TPIGDGP 192
AY015996    rmGFP PVM-QKTILGIEPSFEAMYMN--NGVLVGEVILVYKLNSGKYYSCHMKTL---MKSKGVV 190
AF372525    rrGFP PVM-QQDIVGMQPSYESMYTN--VTSVIGECIIAFKLQTGKHFTYHMRTV---YKSKKPV 187
AF168419    dsRED PVM-QKKTMGWEASTERLYPR--DGVLKGEIHKALKLKDGGHYLVEFKSI---YMAKKP- 186
AF322222 asCP562 P-------------------------------------------------RDAEQS- 137
AF168422 asCP562 PVM-KKMTDNWEPSCEKIIPVPKQGILKGDVSMYLLLKDGGRLRCQFDTV---YKAKSVP 191
AF246709 asFP595 PVM-QNKAGRWEPATEIVYEV--DGVLRGQSLMALKCPGGRHLTCHLHTTYRSKKPASA- 186
AF322221 asFP499 PVM-QKKTCGWEPSTETVIPR--DGGLLLRDTPALMLADGGHLSCFMETT---YKSKKE- 183
AF384683    mcGFP PVM-QKKTLKWEPSTEKMYVR--DGVLKGDVNMALLLEGGGHYRCDFRST---YKAKKR- 182
AF401282    mfGFP PVM-QKKTLKWEPSTEKMYVR--DGVLKGDVNMALLLEGGGHYRCDFKTT---YKAKKF- 185
AF168424 csFP484 PVM-QKKTLKWEPSTEIMYVR--DGVLVGDISHSLLLEGGGHYRCDFKSI---YKAKKV- 224
AF168420 dsFP483 PVV-QKKTTGWEPSTERLYPR--DGVLIGDIHHALTVEGGGHYACDIKTV---YRAKKAA 187
AY015995    spGFP PVM-QKAILGMEPSFEVVYMN--SGVLVGEVDLVYKLESGNYYSCHMKTF---YRSKGGV 190
AF168423 zsFP538 PVM-KKMTTNWEASCEKIMPVPKQGILKGDVSMYLLLKDGGRYRCQFDTV---YKAKSVP 191
AF168421 amFP486 PVM-AKKTTGWDPSFEKMTVC--DGILKGDVTAFLMLQGGGNYRCQFHTS---YKTK-KP 188
AY013824 amGFPXM NILGHKLEYNFNSHNVYIMPDKANNGLKVNFKIRHNIEGGGVQLADHYQTN--VPLGDGP 192

P42212      avGFP VLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK---- 238
AY015996    rmGFP KEFPSYHFIQHRLEKTYV-EDGG-FVEQHETAIAQMTSIGKPLGSLHEWV 238
AF372525    rrGFP ETMPLYHFIQHRLVKTNV-DTASGYVVQHETAIAAHSTIKKIEGSLP--- 233
AF168419    dsRED VQLPGYYYVDSKLDITSH-NEDYTIVEQYERTEGRHHLFL---------- 225
AF322222 asCP562 RKMG----ASHRDTL------------------------------------ 148
AF168422 asCP562 RKMPDWHFIQHKLTREDRSDAKNQKWHLTEHAIASGSALP---------- 231
AF246709 asFP595 LKMPGFHFEDHRIEIMEE-VEKGKCYKQYEAAVGRYCDAAPSKLGHN--- 232
AF322221 asFP499 VKLPELHFHHLRMEKLNI-SDDWKTVEQHESVVASYS-QVPSKLGHN--- 228
AF384683    mcGFP VQLPDYHFVDHRIEILSH-DNDYNTVKLSEDAEARYSMLPSQAK------ 225
AF401282    mfGFP VQLPDYHFVDHRIEILSH-DKDYNKVKLYEHAEA-HSGLPRQAK------ 227
AF168424 csFP484 VKLPDYHFVDHRIEILNH-DKDYNKVTLYENAVARYSLLPSQA------- 266
AF168420 dsFP483 LKMPGYHYVDTKLVIWNN-DKEFMKVEEHEIAVARHHPFYEPKKDK---- 232
AY015995    spGFP KEFPEYHFIHHRLEKTYV-EEGS-FVEQHETAIAQLTTIGKPLGSLHEWV 238
AF168423 zsFP538 SKMPEWHFIQHKLLREDRSDAKNQKWQLTEHAIAFPSALA---------- 231
AF168421 amFP486 VTMPPNHVVEHRIARTDLDKGGNS-VQLTEHAVAHITSVVPF-------- 229
AY013824 amGFPXM VLIPINHYLSTQTAISKDRNETRDHMVFLEFFSACGHTHGMDELYK---- 238
          : :
```

Example 2: Construction of GFP complementation fragment probes

Different fragments of EGFP fused to anti-parallel leucine zippers (called NZ and CZ) that can bind to each other within prokaryotic and eukaryotic cells were used to evaluate the optimal site for splitting EGFP for use of such fragments in molecular complementation experiments, including bimolecular fluorescence complementation experiments. NZ and CZ leucine zippers were prepared by annealing and ligating phosphorylated oligo nucleotides 2110-2115 (for NZ zipper, see Table 2) or phosphorylated oligo nucleotides 2116-2121 (for CZ zipper), into NcoI-BamHI cut pTrcHis-A vector (commercially available from Invitrogen) producing vector PS1515 (expression vector encoding NZ zipper) or PS1516 (expression vector encoding CZ zipper). The oligos ligated in NZ and CZ annealing mixes 1 produced the coding sequences of the N-terminal parts of the NZ and CZ zippers. The oligos ligated in NZ and CZ annealing mixes 2 produced the coding sequences of the middle parts of the NZ and CZ zippers and the oligos ligated in NZ and CZ annealing mixes 3 produced the coding sequences of the C-terminal parts of the NZ and CZ zippers.

Annealing primer pairs for NZ zipper

NZ annealing mix 1

Forward oligo 2110 (1 µM)	5 µl
Reverse oligo 2111 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

NZ annealing mix 2

Forward oligo 2112 (1 µM)	5 µl
Reverse oligo 2113 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

NZ annealing mix 3

Forward oligo 2114 (1 µM)	5 µl
Reverse oligo 2115 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

Each of the annealing mixes was heated at 80°C for 2 minutes on a pre-heated Hybaid OmniGene PCR machine which was subsequently turned off and allowed to cool to room temperature (about 10 min). The fragments were subsequently put on ice.

Annealing primer pairs for CZ zipper

CZ annealing mix 1

Forward oligo 2116 (1 µM)	5 µl
Reverse oligo 2117 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

CZ annealing mix 2

Forward oligo 2118 (1 µM)	5 µl
Reverse oligo 2119 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

CZ annealing mix 3

Forward oligo 2120 (1 µM)	5 µl
Reverse oligo 2121 (1 µM)	5 µl
50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0	2 µl
H_2O	8 µl

Each of the annealing mixes was heated at 80°C for 2 minutes on a pre-heated Hybaid OmniGene PCR machine which was subsequently turned off and allowed to cool to room temperature (about 10 min). The fragments were subsequently put on ice.

Restriction digestion of pTrcHis-A prokaryotic expression vector

The pTrcHis-A prokaryotic expression vector cut with NcoI and BamHI restriction enzymes and gel purified was used for cloning the prepared NZ and CZ leucine zipper coding sequences:

Restriction digestion of pTrcHis-A vector

pTrcHis-A (1 µg/µl)	2 µl
NcoI (10 U/µl)	1 µl
NheI (5 U/µl), optional	0.5 µl
BamHI (20 U/µl)	1 µl
100x BSA	0.4 µl
10x NEB (New England Biolabs, NEB) BamHI buffer	3 µl
H_2O	23 µl
Calf intestinal phosphatase (optional, last 20 min only)	0.5 µl

The vector was digested for about 1 hour at 37°C and purified by agarose gel electrophoresis. The desired vector fragment was recovered from the gel using the QIAquick Gel Extraction kit (spin columns) from Qiagen and recovered in 50 µl of elution buffer. NheI, which cuts between NcoI and BamHI, was included to minimise the amounts of uncut and self-ligating vector.

Ligation and transformation of annealed NZ oligo pairs

Each of the three NZ annealing mixtures 1-3 was diluted 50-fold (1 µl of mixture in 50 µl of H_2O) and mixed and ligated into the cut vector as follows (three hours at 20-24°C):

Ligation of NZ zipper fragments into pTrcHis-A vector

Annealing mix 1	1 µl
Annealing mix 2	1 µl
Annealing mix 3	1 µl
10x T4 DNA ligase buffer (New England Biolabs)	1 µl
T4 DNA ligase (400 U/µl, New England Biolabs)	0.5 µl
pTrcHis-A (NcoI + BamHI cut)	0.5 µl
H_2O	5 µl

Alternatively, the fragments in NZ annealing mixes 1, 2, and 3 can be ligated in absence of vector and purified by agarose gel electrophoresis before being ligated into the NcoI-BamHI cut vector. The annealed and ligated oligo nucleotides from annealing mixes 1-3 had single stranded terminal overhangs that were compatible with the overhangs that were generated by NcoI and BamHI restriction digestion of pTrcHis-A. After ligation of the fragment into cut pTrcHis-A, the NcoI and BamHI sites were regenerated.

Following ligation into the vector, 2 µl of the ligation mixture was transformed into 50 µl of One Shot TOP10 chemically competent E. coli cells (Invitrogen) following the manufacturers protocol. The ligation can be performed using different amounts or volumes fragments and buffers. The inserted DNA sequence and the encoded NZ zipper peptide are as follows:

```
  M  A  G  G  T  G  S  G  A  L  K  K  E  L  Q  A  N  K  K  E
CCATGGCCGGTGGTACCGGTTCCGGTGCCCTGAAGAAGGAGCTGCAGGCCAACAAGAAGGAG

L  A  Q  L  K  W  E  L  Q  A  L  K  K  E  L  A  Q  *  D
CTGGCCCAGCTGAAGTGGGAGCTGCAGGCCCTGAAGAAGGAGCTGGCCCAGTAGGATCC
```

The Gly-Gly-Thr-Gly-Ser-Gly amino acid sequence in the terminus is part of the linker sequence that was inserted between the NZ zipper peptide and the NEGFP fragment (N-terminal fragments of EGFP are called NEGFP). The zipper sequence in the NZ-NEGFP fusion protein is also Gly-Gly-Thr-Gly-Ser-Gly with the Gly-Gly-Thr-Gly coding sequence being repeated in the NEGFP reverse amplification primers 2129, 2130, and 2131 (Table 3). Underlined are the unique NcoI (CCATGG), AgeI (ACCGGT) and BamHI (GGATCC) sites

used for cloning of the zipper peptide into pTrcHis-A and the NZ-NEGFP fragments into the NZ zipper vector PS1515 (see below). The asterisk (*) shows a stop codon.

Ligation and transformation of annealed CZ oligo pairs

Each of the three CZ annealing mixtures 4-6 was diluted 50-fold (1 µl of mixture in 50 µl of H_2O) and mixed as follows:

Ligation of CZ zipper fragments into pTrcHis-A vector

CZ annealing mix 1	1 µl
CZ annealing mix 2	1 µl
CZ annealing mix 3	1 µl
10x T4 DNA ligase buffer (New England Biolabs)	1 µl
T4 DNA ligase (400 U/µl, New England Biolabs)	0.5 µl
pTrcHis-A (NcoI + BamHI cut)	0.5 µl
H_2O	5 µl

Alternatively, the fragments in CZ annealing mixes 1, 2, and 3 can be ligated in absence of vector and purified by agarose gel electrophoresis before being ligated into the NcoI-BamHI cut vector. The annealed and ligated oligo nucleotides from annealing mixes 1-3 had single stranded terminal overhangs that were compatible with the overhangs that were generated by NcoI and BamHI restriction digestion of pTrcHis-A. After ligation of the fragment into cut pTrcHis-A, the NcoI and BamHI sites were regenerated.

Following ligation into the vector, 2 µl of the ligation mixture were transformation into 50 µl of One Shot TOP10 chemically competent E. coli cells (Invitrogen) following the manufacturers protocol. The ligation can be performed using different amounts or volumes fragments and buffers. The inserted DNA sequence and the encoded CZ zipper peptide are as follows:

```
  M  A  S  E  Q  L  E  K  K  L  Q  A  L  E  K  K  L  A  Q  L
CCATGGCCAGCGAGCAGCTGGAGAAGAAGCTGCAGGCCCTGGAGAAGAAGCTGGCCCAGCTG

E  W  K  N  Q  A  L  E  K  K  L  A  Q  G  G  T  G  *
GAGTGGAAGAACCAGGCCCTGGAGAAGAAGCTGGCCCAGGGCGGCACCGGTTAGGATCC
```

The Gly-Gly-Thr-Gly amino acid sequence in the terminus is part of the linker sequence that was inserted between the CZ zipper peptide and the CEGFP fragment (C-terminal fragments of EGFP are called CEGFP). The zipper sequence in the CZ-CEGFP fusion protein is also Gly-Gly-Thr-Gly with the Thr-Gly coding sequence being repeated in the CEGFP forward amplification primers 2133, 2134, and 2135 (Table 3). Underlined are the unique NcoI (CCATGG), AgeI (ACCGGT) and BamHI (GGATCC) sites used for cloning of the zipper peptide into pTrcHis-A and the CZ-CEGFP fragments into the CZ zipper vector PS1516 (see below). The asterisk (*) shows a stop codon.

Example 3: E. coli colony PCR screen, plasmid miniprep and DNA sequencing

The transformed bacteria were plated on Luria Broth (LB) agar plates containing 100 µg/ml of carbenicillin as selection (present in used E. coli media). To quickly identify transformants containing the desired NZ or CZ constructs, colony PCR screening was performed using oligos 2110 (5' forward NZ oligo) and 2115 (3' reverse NZ oligo) or using oligos 2116 (5' forward CZ oligo) and 2121 (3' reverse CZ oligo):

Per sample (15 µl reaction volume)

10x Taq polymerase buffer (Perkin Elmer)	1.5 µl
dNTP (5 mM nucleotide, each)	0.3 µl
50 mM $MgCl_2$	0.6 µl
Dimethyl sulphoxide (DMSO)	0.3 µl
Taq polymerase (Perkin Elmer)	0.2 µl
5' forward primer (10 µM)	0.5 µl
3' reverse primer (10 µM)	0.5 µl
H_2O	6.1 µl
Transformant resuspended in H_2O	5.0 µl

Cycling parameters (RoboCycler Gradient 96, Stratagene)

Initial denaturation at 94°C for 3 min followed by 25 cycles of (all steps of 1 min):

Denaturation at 94°C, primer annealing at 53°C and primer extension at 72°C.

Finally, an additional extension step at 72°C was included (5 min).

16 NZ transformants and 16 CZ transformants were screened. PCR fragments having the expected product sizes of about 120 base pairs were amplified from 14 NZ clones and 15 CZ clones, as determined by agarose gel electrophoresis analysis.

Three of the positive colonies were picked from each transformation (NZ and CZ) and used to inoculate 5 ml of liquid LB medium. After culturing at 37°C over night, plasmid DNA was purified by mini preparations using the QIAprep kit from Qiagen.

Plasmids containing correct NZ (PS1515) or CZ (PS1516) fragment inserts were identified by DNA sequencing on an ABI PRISM model 377 DNA sequencer using forward sequencing primer 1282.

Example 4: Prokaryotic expression vectors encoding EGFP fragment/zipper fusion proteins

The DNA sequences encoding the NZ and CZ zippers in the prokaryotic expression vectors PS1515 and PS1516, respectively, can be fused to DNA sequences encoding desired EGFP fragments (N-terminal fragments of EGFP are called NEGFP and C-terminal fragments of EGFP are called CEGFP) or other fragments using the unique AgeI restriction sites appropriately located in linker sequences in either the 5' end (as in the NZ vector PS1515) or in the 3' end (as in the CZ vector PS1516) of the leucine zipper coding sequence in combination with either of the unique NcoI or BamHI sites used for cloning the zipper coding fragments (DNA and amino acid sequences are shown above). The general structures of the fusion protein coding sequences are shown in Figure 1.

For example, to prepare a prokaryotic expression vector encoding a fusion protein consisting of NZ zipper N-terminally fused to an NEGFP fragment, e.g. residues 1-172, this region of the EGFP coding sequence in the commercial expression vector pEGFP-C1 (Clontech) was amplified by PCR using forward oligo 2128 (containing a unique NcoI site) and reverse oligo 2131 (containing a unique AgeI site) in accordance with Table 3.

Per sample (50 µl reaction volume)

10x Pfu polymerase buffer (Stratagene)	5.0 µl
dNTP (5 mM nucleotide, each)	1.0 µl
Pfu Hot Start polymerase (Stratagene)	1.0 µl
5' forward primer (10 µM)	1.0 µl
3' reverse primer (10 µM)	1.0 µl
pEGFP-C1 vector (10 ng/µl)	2.0 µl
H_2O	39.0 µl

Cycling parameters (Hybaid OmniGene PCR machine)

Initial denaturation at 94°C for 3 min followed by 25 cycles of (all steps of 1 min):

Denaturation at 94°C, primer annealing at 53°C and primer extension at 72°C.

Finally, an additional extension step at 72°C was included (5 min).

The PCR fragment encoding the desired EGFP fragment, e.g. the above mentioned fragment composed of residues 1-172, with appropriately engineered terminal restriction sites contained in the primer sequences was then gel purified as described above cut with NcoI and AgeI or AgeI and BamHI and ligated into the constructed NZ or CZ prokaryotic leucine zipper expression vectors PS1515 or PS1516 cut with the same enzymes and gel purified:

Restriction digestion of NEGFP and CEGFP PCR fragments

EGFP fragment (gel purified)	26 µl
NcoI (10 U/µl) or BamHI (20 U/µl)	0.5 µl
AgeI (10 U/µl)	1.0 µl
10x New England Biolabs buffer 2	3

Restriction digestion of NZ (PS1515) and CZ (PS1516) vectors

Vector (1 µg/µl)	1.0 µl
NcoI (10 U/µl) or BamHI (20 U/µl)	0.33 µl
AgeI (10 U/µl)	0.66 µl
10x New England Biolabs buffer 2	1
H_2O	7

All enzymes were from New England Biolabs. The DNA preparations were digested for 1 hour at 37°C and gel purified.

Ligation of EGFP fragments into cut PS1515 or PS1516 vector

Cut and purified vector	2 µl
Cut and purified NEGFP or CEGFP fragment	4 µl
10x T4 DNA ligase buffer (New England Biolabs)	1 µl
T4 DNA ligase (400 U/µl, New England Biolabs)	0.5 µl
H_2O	2.5 µl

Ligation proceeded for 30 min at 22°C after which 2 µl of each ligation mixture were transformed into 50 µl of One Shot TOP10 chemically competent E. coli cells (Invitrogen). The transformed cells were plated on LB plates containing carbenicillin and plasmids were prepared from two colonies from each transformation as described above.

Example 5: EGFP based bimolecular fluorescence complementation in E. coli

Plasmids that expressed functional NZ-NEGFP or CZ-CEGFP complementation constructs were identified by co-transforming 10 µl of One Shot TOP10 chemically competent E. coli cells (Invitrogen) with 1 µl of each of appropriately matched NZ-NEGFP or CZ-CEGFP plasmids (i.e., plasmids that express EGFP fragments, said fragments are truncated after (NEGFP fragments) or before (CEGFP fragments) the same splitting site and plating the co-transformed cells on LB plates containing carbenicillin and 5 mM of isopropyl-ß-thiogalactoside (IPTG).

The transformed cells were grown over night at 37°C. E. coli colonies that were green fluorescent because of EGFP based bimolecular fluorescence complementation were visible on the agar plate without magnification about 10-20 hours after transfection (the fluorescence developed further during storage of the plates at 5°C for one or more days) when illuminated with a blue light source (Fiberoptic-Heim LQ2600) and viewed through yellow filter glasses.

Functional complementation was clearly visible in cells co-transformed with complementation constructs based on splits between either residues 157 and 158 or between residues 172 and 173 and the DNA sequences of expression vectors that produced functional NZ-NEGFP or CZ-CEGFP complementation fragments (named PS1594, PS1595, PS1596, PS1597, see Table 4) were verified by DNA sequencing using primer 1282 as previously described.

Surprisingly, the E. coli colonies of cells co-transformed with the vectors expressing the EGFP complementation fragments with split in the Ile171-Ser175 loop (namely between residues 172 and 173, vectors PS1595 and PS1597) were significantly more fluorescent than the colonies of cells that were co-transformed with vectors expressing EGFP complementation fragments that were split in the Ala154-Gly160 loop (namely between residues 157 and 158, vectors PS1594 and PS1596).

Functional complementation was not clearly visible in cells co-transformed with complementation constructs based on a split between residues 144 and 145. DNA sequencing confirmed that expression vectors PS1614 and PS1615 encoded the correct NZ-NEGFP and CZ-CEGFP complementation fragments, respectively.

Example 6: Eukaryotic expression vectors encoding EGFP fragment/zipper fusion proteins

Because of the low fluorescence signal produced by the complementation fragments based on the 144/145 split fragments, only the complementation fragments that were based on splits at residues 157/158 or 172/173 were transferred to a eukaryotic expression system to permit evaluation of fragment complementation in mammalian cells.

NZ-NEGFP fragments in PS1596 and PS1597, and CZ-CEGFP fragments in PS1594 and PS1595, are flanked by an NcoI site 5' to the start codons and a BamHI site 3' to the stop

codons. The fragments were transferred as blunt-ended NcoI/BamHI fragments into mammalian expression vectors cut with Eco47III/BamHI. To select for stable expression of both an NZ-NEGFP and a CZ-CEGFP expressing plasmid, the expression vectors for NZ-NEGFP fragments and CZ-CEGFP fragments contain selection markers for neomycin/geneticin/G418 and zeocin, respectively.

Plasmids PS1594, PS1595, PS1596, and PS1597 were cut with NcoI restriction enzyme, blunt-ended with Klenow fragment, gel purified, cut with BamHI and gel purified as described below.

Restriction digestion of NZ-NEGFP and CZ-CEGFP prokaryotic expression vectors

PS1594, PS1595, PS1596, or PS1597 (1 µg/µl)	1 µl
NcoI (10 U/µl, from New England Biolabs)	1 µl
10x buffer 4 (NEB)	3 µl
H_2O	25 µl

The plasmids were digested for about 1 hour at 37°C. 1 µl of 1 mM dNTP mix and 1 unit of Klenow fragment (New England Biolabs) were added and the reactions were incubated 30 minutes at room temperature. The linear plasmid fragments were purified by agarose gel electrophoresis and recovered from the gel using the QIAquick Gel Extraction kit (spin columns) from Qiagen and recovered in 50 µl of elution buffer. 5 µl BamHI buffer (New England Biolabs) and 10 units BamHI enzyme were added. The plasmids were digested for about 1 hour at 37°C. The desired plasmid fragments were purified by agarose gel electrophoresis and recovered from the gel using the QIAquick Gel Extraction kit (spin columns) from Qiagen and recovered in 50 µl of elution buffer.

To stably co-express NZ-NEGFP and CZ-CEGFP fragments in the same mammalian cell, mammalian expression vectors carrying different selection markers were required. To obtain this, the kanamycin/neomycin selection marker on the expression vector pEGFP-C1 was replaced with a zeocin resistance marker resulting in the plasmid referred to as PS0609.

Replacement of kanamycin/neomycin marker on pEGFP-C1 with zeocin marker.

pEGFP-C1 was digested with AvrII, which excises the kanamycin/neomycin selection marker, and following gel purification, the vector fragment was ligated with an approximately 0.5 kbp AvrII fragment encoding zeocin resistance. This fragment was isolated by PCR amplification of the zeocin selection marker on plasmid pZeoSV (Invitrogen) using primers 9655 and 9658 (see Table 2). Both primers contain AvrII cloning sites and flank the zeocin resistance gene on plasmid pZeoSV including its E. coli promoter. The top primer 9658 spans the AseI site at the beginning of zeocin, which can be used to determine the orientation of the AvrII insert relative to the SV40 promoter which drives resistance in mammalian cells. The resulting plasmid is referred to as PS0609.

Plasmids pEGFP-C1 (Clontech) and its zeocin-resistant derivative PS0609 were cut with Eco47III restriction enzyme, gel purified, cut with BamHI and gel purified as described below. These steps excise EGFP and leave the rest of the vectors intact.

Restriction digestion of eukaryotic expression vectors

pEGFP-C1 or PS0609 DNA (1 µg/µl)	0.5 µl
Eco47III (10 U/µl, from Promega)	1 µl
10x buffer D (Promega)	3 µl
H_2O	25.5 µl

The plasmids were digested for about 1 hour at 37°C. The linear plasmid fragments were purified by agarose gel electrophoresis and recovered from the gel using the QIAquick Gel Extraction kit (spin columns) from Qiagen and recovered in 50 µl of elution buffer. 5 µl BamHI buffer (New England Biolabs) and 10 units BamHI enzyme were added. The plasmids were digested for about 1 hour at 37°C. The desired vector fragments were purified by agarose gel electrophoresis and recovered from the gel using the QIAquick Gel Extraction kit (spin columns) from Qiagen and recovered in 50 µl of elution buffer.

Ligation of NZ-NEGFP fragments into pEGFP-C1 and CZ-CEGFP fragments into PS0609

Cut and purified vector fragment	1 µl
Cut and purified NZ-NEGFP or CZ-CEGFP fragment	3 µl
10x T4 DNA ligase buffer (New England Biolabs)	1 µl
T4 DNA ligase (400 U/µl, New England Biolabs)	0.5 µl
H2O	5 µl

Ligation reactions were incubated at 16°C overnight. 3 µl were transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen) and transformants were selected on imMedia with kanamycin or imMedia with zeocin (both from Invitrogen) for pEGFP-C1 and PS0609 derivatives, respectively.

4 transformants from each transformation plate were picked in imMedia medium with appropriate selection (kanamycin or zeocin) and grown at 37 degrees C for 6 hours. Plasmid DNA was isolated by the QIAprep spin column method (Qiagen) and analysed by restriction digests with AseI and MluI. The DNA sequences of the inserts were finally verified by sequencing as described above. The resulting plasmids were named PS1557, PS1558, PS1559, and PS1560 (Table 4).

Example 7: EGFP based bimolecular fluorescence complementation in mammalian cells

To establish cells lines that express EGFP fragment/zipper fusion proteins, CHO-hIR cells were transfected with plasmid pairs resulting in two cell lines 1) CHO-hIR PS1559+PS1557, and 2) CHO-hIR PS1560+PS1558. The CHO-hIR cell line consists of CHO-K1 (ATCC CCL-61) cells that have been stably transfected with the human insulin receptor (hIR, GenBank Acc# M10051) as described in: Hansen, B. F., Danielsen, G. M., Drejer, K., Sørensen, A. R., Wiberg, F. C., Klein, H. H., Lundemose, A. G. (1996) Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. Biochem. J. Apr 1; 315 (Pt 1):271-279. The selection marker for the vector is methotrexate (MTX). The hIR expression is very stable in the CHO-hIR cells, without selection pressure, because of the insulin-sensitivity of the cell line and a very stable phenotype can be maintained without selection pressure.

Stable cells were obtained by cell growth in selection medium containing Geneticin and Zeocin.

CHO-hIR cells were transfected using Fugene (Roche) according to the manufacturer's instructions. The day after transfection, cells were examined for transient expression, split 1:10 and exposed to selection medium (growth medium supplemented with 500 µg/ml geneticin (Invitrogen) and 1 mg/ml zeocin (Cayla). The cells lines were stable after 2-3 weeks of culture in selection medium.

The growth medium used was NUT.MIX F-12 (Ham's) with GLUTAMAX-1 (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosciences) and 1% Penicillin-Streptomycin (10,000 IU/ml, Gibco/Invitrogen). The CHO-hIR cells were cultured in growth medium, and split 1:4 to 1:16 twice a week according to standard cell culture protocols. The CHO-hIR PS1559+PS1557 and CHO-hIR PS1560+PS1558 were treated likewise, except that the growth medium was supplemented with 500 µg/ml geneticin (Invitrogen) and 1 mg/ml zeocin (Cayla) at all times.

Images of three CHO cell lines separately transfected with pEGFP-C1 (expressing EGFP with a short C-terminal extension), PS1559 + PS1557 (expressing EGFP complementation fragments split at 157-158, CG157 + NG158) and with PS1560 + PS1558 (expressing EGFP complementation fragments split at 172-173, CG172 + CN173) were collected 1 day, 2 days and 10 days after transfection to assess the relative brightness of cells expressing the different complementation constructs. Images were collected on a Nikon Diaphot 300 equipped for epifluorescence work. Light source for epifluorescence was a Nikon 100W Hg arc lamp, coupled to the microscope through a custom quartz fibre illuminator (TILL Photonics GmbH, Planegg, Germany). Excitation light passed through a 450-490 nm bandpass filter (Delta Light and Optics, Lyngby, Denmark) and was directed to the specimen via a Chroma 72100 505 nm cut-on dichroic mirror (Chroma Technology, Brattleboro, VT, USA). A x40 NA1.3 oil immersion lens was used for all images. Emitted light passed through a 540-550 bandpass filter (Chroma) to a Hammamatsu Orca ER camera. All images were collected with 50 millisecond exposure time, chosen to ensure non-saturation of images for even the brightest (EGFP-expressing) cells in each optical field (maximum pixel count <4095). Imaging software used to acquire images on this system was IPLab for Windows (Scanalytics, USA).

Presentation and analysis of images

The microscope images were analysed using the ImageJ software package, the public domain image analysis software written by Wayne Rasband of the US National Institute of Health (http://rsb.info.nih.gov/ij/) and the data analysis was performed in Microsoft Excel. The images shown in Figure 2 are of fluorescent CHO-hIR cells co-transfected with different NZ-NEGFP (NG) and CZ-CEGFP (CG) expression vectors or transfected with pEGFP-C1. The images are scaled individually to visualise the cells and the fluorescence distribution within them. Because of this scaling, the relative fluorescence levels cannot be compared between the images. When the same images are scaled identically they appear as in Figure 3 and it is apparent that the cells that are transfected with complementation constructs that are based on a split between residues 172 and 173 are significantly more fluorescent than the cells that are transfected with complementation constructs that are based on a split between residues 157 and 158. However, the cells transfected with the pEGFP-C1 construct show significantly stronger fluorescence on day 2.

The same images were analysed for background and maximum fluorescence intensities using the ImageJ software package (Figure 4). From the figure, it is clear that a split between residues 172 and 173, and probably anywhere else in this loop, is greatly superior to a split between residues 157 and 158 and probably also to splits anywhere else in this loop.

Example 8: Eukaryotic expression vectors encoding EYFP and EYFP variant F64L fragment/zipper fusion proteins

In the following examples, this nomenclature will be used:

Y	Fragment of EYFP
G	Fragment of EGFP
N157	N-terminal fluorescent protein fragment C-terminally truncated by a split in or close to the loop between beta sheets 7 and 8 (residues 154-160), e.g. between residues 154 and 155 or between residues 157 and 158 in EGFP or EYFP. The fragment is fused to a leucine zipper sequence as described in Example 4.
N172	N-terminal fluorescent protein fragment C-terminally truncated by a split in or close to the loop between beta sheets 8 and 9 (residues 171-175), e.g. between residues 172 and 173 in EGFP or EYFP. The fragment is fused to a leucine zipper sequence as described in Example 4.

C158 C-terminal fluorescent protein fragment N-terminally truncated by a split in or close to the loop between beta sheets 7 and 8 (residues 154-160), e.g. between residues 154 and 155 or between residues 157 and 158. The fragment is fused to a leucine zipper sequence as described in Example 4.

C173 C-terminal fluorescent protein fragment N-terminally truncated by a split in or close to the loop between beta sheets 8 and 9 (residues 171-175), e.g. between residues 172 and 173 in EGFP or EYFP. The fragment is fused to a leucine zipper sequence as described in Example 4.

F64L Fluorescent protein variant containing a leucine residue in position 64, e.g. in place of a phenyl alanine residue. This residue is in position 65 in EGFP and EYFP because of an extra residue (Val2) in position 2.

Mutagenesis of the eukaryotic NG expression vectors PS1559 (NG157) and PS1560 (NG172) into the corresponding N-terminal EYFP (SEQ ID NO: 5) fragment (NY) variants and mutagenesis of the eukaryotic CG expression vectors PS1557 (CG158) and PS1558 (CG173) into the corresponding C-terminal EYFP fragment (CY) variants was accomplished by site directed mutagenesis using the QuickChange kit and by following the manufactorers instructions (Stratagene). Primers 2333 and 2334 were used to convert expression vectors PS1559 (NG157) and PS1560 (NG172) into N-terminal EYFP fragment expression vectors PS1639 (NY157) and PS1642 (NY172). The introduced mutations were: L64F:T65G:V68L:S72A. Furthermore, primers 2335 and 2336 were used to convert expression vectors PS1559 (NG157) and PS1560 (NG172) into F64L mutated N-terminal EYFP fragment expression vectors PS1640 (NY157 F64L) and PS1641 (NY172 F64L). The introduced mutations were: T65G:V68L:S72A. Accordingly, the expressed YN fragments have the following amino acid sequences (only residues 64-72 are shown):

NG (template)	L64	T65	Y66	G67	V68	Q69	C70	F71	S72
NY (L64F:T65G:V68L:S72A)	F64	G65	Y66	G67	L68	Q69	C70	F71	A72
NY F64L (T65G:V68L:S72A)	L64	G65	Y66	G67	L68	Q69	C70	F71	A72

Finally, primers 2337 and 2338 were used to convert expression vectors PS1557 (CG158) and PS1558 (CG173) into C-terminal EYFP fragment expression vectors PS1637 (CY158) and PS1638 (NY173) by introducing a T203Y mutation. All sequences were

verified by DNA sequencing of the vectors and all primer sequences are shown in Table 2.

Example 9: EGFP based bimolecular fluorescence complementation in mammalian cells

The constructed EYFP based split fluorescent protein expression vectors PS1637 to PS1642 described above were investigated in mammalian cells in parallel with the EGFP based split fluorescent protein expression vectors PS1557 to PS1560 described in Example 7 and using the same experimental set-up (including the same filter set) and procedures (including the image analysis procedure) except that all images were produced using 10 ms exposure times instead of 50 ms exposure times, because of the increased brightness of the probes, and a 20x objective was used instead of a 40x objective to image more cells. Other appropriate filter sets could have been used. The images are taken the day after transfection (day 1).

It is apparent from the identically scaled fluorescence images of the transfected cells (Figure 6) that the split site between residues 172 and 173 is again shown to be superior to the split site between residues 157 and 158. Furthermore, it is apparent that complementation based on EYFP fragments is superior to complementation based on EGFP fragments. Surprisingly, introduction of the F64L mutation from EGFP into the N-terminal EYFP fragments further greatly enhanced the fluorescence of the complementing fragments. As can be seen from the images, the positive effects of using the optimal splitting site (between residues 172 and 173) using the optimal fluorescent protein colour variant (EYFP) and introducing the F64L folding mutation into the NY fragment, are additive. Quantification of these observation was done by analysing the images shown in Figure 6 and the numeric out-put is presented in Figure 7.

Effects of colour (yellow better):

Good		Better
EGFP	vs	EYFP
GN157 + GC158	vs	YN157 + YC158
GN172 + GC173	vs	YN172 + YC173

Effects of split site (172/173 better):

Good		Better
GN157 + GC158	vs	GN172 + GC173
YN157 + YC158	vs	YN172 + YC173
GN157 F64L + GC158	vs	YN172 F64L + YC173

Effects of F64L (+F64L better):

Good		Better
YN157 + YC158	vs	YN157 F64L + YC158
YN172 + YC173	vs	YN172 F64L + YC173

It is interesting to note, that the optimal constructs (YN172 F64L + YC173) when re-assembled is nearly as intense as EYFP itself. The great increase in fluorescence intensity is important in many types of quantitative cell analyses (e.g. high through-put screening and microscopy) to increase the signal to noise rations, to facilitate detection of low amounts of probes in vivo or in vitro, etc.

Mixing YN and GC or GN and YC fragments can also produce functional fluorescent complexes, potentially of different colours (Figs. 8 and 9). Fragments having overlapping sequences are also functional and may be very attractive in e.g. functional cloning systems where highly flexible linkers sequences are required due to the very diverse nature of the fusion partners. The overlapping fragments permit either of the fusion partners to have a long linker sequence (Figure 8, quantified in Figure 9).

Figure legends

Figure 1

General structures of the fusion protein coding sequences.

Figure 2

16 bit images of fluorescent CHO-hIR cells co-transfected with NZ-NEGFP and CZ-CEGFP expression vectors or transfected with pEGFP-C1 were taken and scaled individually to visualise the cells and the fluorescence distribution within them. Because of the pixel intensity scaling, the relative fluorescence levels cannot be compared among the images. The splitting sites are either at residues 157/158 (top row, plasmids PS1557 and PS1559) or at residues 172/173 (middle row, plasmids PS1558 and PS1560). The EGFP expression vector pEGFP-C1 was transfected into the cells in the bottom row. The images were taken 1 day (left column), 2 days (middle column), or 10 days (right column) after transfection. The images of the cells are representative of the cells that expressed functionally complementing fragments.

Figure 3

The same 16 bit images of fluorescent CHO-hIR cells co-transfected with NZ-NEGFP and CZ-CEGFP expression vectors or transfected with pEGFP-C1 as shown in Figure 2 but the images are now shown with the same intensity scaling to allow comparison of fluorescence intensities. The cells that are transfected with complementation constructs that are based on a split between residues 172 and 173 (middle row) are clearly more fluorescent than the cells that are transfected with complementation constructs that are based on a split between residues 157 and 158 (top row). However, the cells transfected with the pEGFP-C1 construct (bottom row) show significantly stronger fluorescence at day 2.

Figure 4

The un-manipulated microscope images shown in Figure 3 were analysed using the ImageJ software package and data analysis was performed in Microsoft Excel. For each 16-bit monochrome IP Lab microscope image, pixel intensity data were produced in

ImageJ and exported to an Excel spread-sheet for data analysis. The darkest and brightest 0.5% of the pixels were identified in each image and the average intensities of these two groups of pixels were calculated. The average intensity of the 0.5% darkest pixels was defined as the back ground fluorescence intensity (shown as white bars in the histogram) and the intensity of the 0.5% brightest pixels was defined as the maximum intensity. The difference in intensity between the maximum intensity and the background intensity was defined as the response (shown as cross hatched bars in the histogram). The sum of the background intensity and the response is equal to the maximum intensity. From the figure, it is clear that EGFP based fluorescence complementation using a split between residues 172 and 173, and probably anywhere else in this loop, is greatly superior to EGFP based fluorescence complementation using a split between residues 157 and 158 and probably also to splits anywhere else in this loop.

Figure 5

Positions of appropriate fluorescent protein splitting sites are shown on ribbon and wire frame representations of GFP. The two representations show the same sites from two sides (molecule rotated approximately 180 degrees around a vertical axis).

Figure 6

Co-transfection of expression vectors expressing EGFP and EYFP based complementation fragments as described in Figure 3 to compare the abilities of the various complementation fragments to combine in cells and produce functional complexes. All images are scaled identically to allow direct comparison of fluorescence intensities between the images.

Single transfections with N-terminal fragments only resulted in no detectable fluorescence above the back ground level (data not shown). These N-terminal fragments contains the chromophore.

Figure 7

Quantitative analysis of the images shown in Figure 6. The results are in accord with the impressions from visual inspection of the cells. The data were produced as described in the legend to Figure 4.

Figure 8

Co-transfection of expression vectors expressing EGFP and EYFP based complementation fragments as described in Figure 3 to compare the effects of mixing differently coloured EGFP, EYFP and EYFP F64L fragments and to determine the influence of overlapping fragments, e.g. combining fragments encoding residues 1-172 and 158-238. All colour combinations complement but typically less efficient than in the correct combinations. Fragments having overlapping regions are also functional and this may be avantagous in experiments where longer linker sequences are or may be required by the fusion partners due to steric hinderance. This was not the case in this experiments where the fusion partners are leucine zippers. In the example (middle column), residues 158-172 were present in both fragments. In all situations, the F64L has a favourable effect on the fluorescence intensities. All images are scaled identically to allow direct comparison of fluorescence intensities between the images.

Figure 9

Quantitative analysis of the images shown in Figure 8. The results can be compared directly with the results shown in Figure 7 and they are in accord with the impressions from visual inspection of the cells. The data were produced as described in the legend to Figure 4.

Tables

Table 2 Oligo nucleotides used in cloning. Oligo nucleotides beginning with P* are phosphorylated at the 5' end to permit ligation.

Oligo nucleotide	Oligo nucleotide sequence (5' end to 3' end)
1282	CAGACAATCTGTGTGGGCACTCGACCGG
2110	P*CATGGCCGGTGGTACCGGTTCCGGTGCCCTGAAGAAGGAGCTGCAGG
2111	P*AGCTCCTTCTTCAGGGCACCGGAACCGGTACCACCGGC
2112	P*CCAACAAGAAGGAGCTGGCCCAGCTGAAGTGGGAGCTGCAG
2113	P*CTCCCACTTCAGCTGGGCCAGCTCCTTCTTGTTGGCCTGC
2114	P*GCCCTGAAGAAGGAGCTGGCCCAGTAG
2115	P*GATCCTACTGGGCCAGCTCCTTCTTCAGGGCCTGCAG
2116	P*CATGGCCAGCGAGCAGCTGGAGAAGAAGCTGCAGGCCCTG
2117	P*CCTGCAGCTTCTTCTCCAGCTGCTCGCTGGC
2118	P*GAGAAGAAGCTGGCCCAGCTGGAGTGGAAGAACCAGGCCCTGGAG
2119	P*GGCCTGGTTCTTCCACTCCAGCTGGGCCAGCTTCTTCTCCAGGG
2120	P*AAGAAGCTGGCCCAGGGCGGCACCGGTTAG
2121	P*GATCCTAACCGGTGCCGCCCTGGGCCAGCTTCTTCTCCAG
2128	GGCGCCATGGTGAGCAAGGGCGAG
2129	GCCGGACCGGTACCACCGTTGTACTCCAGCTTGTG
2130	GCCGGACCGGTACCACCCTGCTTGTCGGCCATG
2131	GCCGGACCGGTACCACCCTCGATGTTGTGGCGGATC
2132	CCCCGGATCCTACTTGTACAGCTCGTCCATGC
2133	GGCGCCATGGGCACCGGTTACAACAGCCACAACGTC
2134	GGCGCCATGGGCACCGGTAAGAACGGCATCAAGGTG
2135	GGCGCCATGGGCACCGGTGACGGCAGCGTGCAGCTC
2333	GCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGC TACCCCGACCACATG
2334	CATGTGGTCGGGGTAGCGGGCGAAGCACTGCAGGCCGTAGCCGAAGGT GGTCACGAGGGTGGGC
2335	GCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGC TACCCCGACCACATG
2336	CATGTGGTCGGGGTAGCGGGCGAAGCACTGCAGGCCGTAGCCCAGGGT GGTCACGAGGGTGGGC
2337	GACAACCACTACCTGAGCTACCAGTCCGCCCTGAGC
2338	GCTCAGGGCGGACTGGTAGCTCAGGTAGTGGTTGTC
9655	TCCTAGGTCAGTCCTGCTCCTCGGCCACGAAGTGCAC TCCTAGGCTGCAGCACGTGTTGACAATTAATCATCGG
9658	CAGACAATCTGTGTGGGCACTCGACCGG

Table 3 Primer pairs used in EGFP fragment amplification

Protein encoded by PCR fragment	5' primer	3' primer
EGFP(1-144)	2128	2129
EGFP(1-157)	2128	2130
EGFP(1-172)	2128	2131
EGFP(145-238)	2133	2132
EGFP(158-238)	2134	2132
EGFP(173-238)	2135	2132

Table 4 Cloning and expression vectors

Vector	Expressed protein	Promotor	Selection E.coli/mamm.
pEGFP-C1	EGFP	CMV	kan/neo
PS0609	EGFP	CMV	zeo/zeo
pTrcHis-A	no insert	Trc	amp/none
PS1515	NZ leucine zipper	Trc	amp/none
PS1516	CZ leucine zipper	Trc	amp/none
PS1614	NZ-NEGFP(1-144)	Trc	amp/none
PS1596	NZ-NEGFP(1-157)	Trc	amp/none
PS1597	NZ-NEGFP(1-172)	Trc	amp/none
PS1615	CZ-CEGFP(145-238)	Trc	amp/none
PS1594	CZ-CEGFP(158-238)	Trc	amp/none
PS1595	CZ-CEGFP(173-238)	Trc	amp/none
PS1559	NZ-NEGFP(1-157)	CMV	kan/neo
PS1560	NZ-NEGFP(1-172)	CMV	kan/neo
PS1557	CZ-CEGFP(158-238)	CMV	zeo/zeo
PS1558	CZ-CEGFP(173-238)	CMV	zeo/zeo

Sequences

SEQ ID 1 Amino acid sequence of GFP

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF
KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY
LSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

SEQ ID 2 Amino acid sequence of GFP Y66W

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTFSWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF
KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY
LSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

SEQ ID 3 Amino acid sequence of GFP Y66H

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTFSHGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF
KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY
LSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

SEQ ID 4 Amino acid sequence of EGFP

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICT
TGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN
VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNH
YLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

SEQ ID 5 Amino acid sequence of EYFP

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICT
TGKLPVPWPTLVTTFGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN
VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNH
YLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

SEQ ID 6 Amino acid sequence of EYFP F64L variant

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICT

TGKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN
VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNH
YLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

Claims

1. Two GFP fragments comprising
(a) an N-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number 1 to amino acid number X of GFP, wherein the peptide bond between amino acid number X and amino acid number X+1 is within a loop of GFP and
(b) a C-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number X+1 to amino acid number 238 of GFP.

2. Two GFP fragments comprising
(a) an N-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number 1 to amino acid number X of GFP, wherein the peptide bond between amino acid number X and amino acid number X+1 is within a loop of GFP and
(b) a C-terminal fragment of GFP, comprising a continuous stretch of amino acids from amino acid number Y to amino acid number 238 of GFP, wherein Y<X creating an overlap of the two GFP fragments, and wherein the peptide bond between amino acid Y-1 and amino acid Y is within a loop of GFP.

3. Two GFP fragments according to the preceding claim, wherein GFP is selected from the group consisting of EGFP, EYFP, ECFP, dsRed and Renilla GFP.

4. Two GFP fragments according to any of the preceding claims, wherein the GFP is EGFP.

5. Two GFP fragments according to any of the preceding claims, wherein the GFP is EYFP.

6. Two GFP fragments according to any of the preceding claims, wherein the amino acid in position 1 preceding the chromophore has been mutated to provide an increase of fluorescence intensity.

7. Two GFP fragments according to the preceding claim, wherein the amino acid F in position 1 preceding the chromophore has been substituted by L.

8. Two GFP fragments according to any of the preceding claims, wherein the GFP has been mutated to further contain the S72A mutation.

9. Two GFP fragments according to any of the preceding claims, wherein X is between 9 and 10 within the Thr9-Val11 loop; or between 23 and 24 within the Asn23-His25 loop; or between 38 and 39 within the Thr38-Gly40 loop; or between 48 and 55 within the Cys48-Pro56 loop; or between 72 and 75 within the Ser72-Asp76 loop; or between 81 and 82 within the His81-Phe83 loop; or between 88 and 89 within the Met88-Glu90 loop; between 101 and 102 within the Lys101-Asp103 loop; or between 114 and 117 within the Phe114-Thr118 loop; or between 128 and 144 within the Ile 128-Tyr145 loop; or between 154 and 159 within the Ala154-Gly160 loop; or between 171 and 174 within the Ile171-Ser175 loop; or between 188 and 196 within the Ile188-Asp197 loop; or between 210 and 214 within the Asp210-Art215 loop.

10. Two GFP fragments according to the preceding claim, wherein X is between 154 and 159 within the Ala154-Gly160 loop.

11. Two GFP fragments according to the preceding claim, wherein X is 157 within the Ala154-Gly160 loop.

12. Two GFP fragments according to the preceding claim, wherein X is between 171 and 174 within the Ile171-Ser175 loop.

13. Two GFP fragments according to any of the preceding claims, wherein X is 172 within in Ile171-Ser175 loop.

14. Two GFP fragments according to the preceding claim, wherein Y is between 154 and 159 within the Ala154-Gly160 loop.

15. Two GFP fragments according to the preceding claim, wherein Y is 157 within the Ala154-Gly160 loop.

16. Two GFP fragments according to any of the preceding claims, wherein the N-terminal fragment of GFP is fused in frame with a first protein of interest.

17. Two GFP fragments according to any of the preceding claims, wherein the first protein of interest is fused to the N-terminal of the N-terminal fragment of GFP

18. Two GFP fragments according to any of the preceding claims, wherein the first protein of interest is fused to the C-terminal of the N-terminal fragment of GFP.

19. Two GFP fragments according to any of the preceding claims, wherein the C-terminal fragment of GFP is fused in frame with a second protein of interest.

20. Two GFP fragments according to any of the preceding claims, wherein the second protein of interest is fused to the N-terminal of the C-terminal fragment of GFP.

21. Two GFP fragments according to any of the preceding claims, wherein the second protein of interest is fused to the C-terminal of the C-terminal fragment of GFP.

22. Two GFP fragments according to any of the preceding claims, wherein the N-terminal fragment of GFP fused in frame to a first protein of interest further comprises a linker sequence between the N-terminal fragment of GFP and the first protein of interest.

23. Two GFP fragments according to any of the preceding claims, wherein the C-terminal fragment of GFP fused in frame to a second protein of interest further comprises a linker sequence between the C-terminal fragment of GFP and the second protein of interest.

24. Two GFP fragments according to any of the preceding claims, wherein the GFP is EYFP further containing an F64L mutation, wherein X is 172, wherein the first protein of interest fused to the N-terminal fragment of GFP is fused to the C-terminal of the N-terminal fragment of GFP and wherein the second protein of interest fused to the C-terminal fragment of GFP is fused to the N-terminal of the C-terminal fragment of GFP.

25. Two GFP fragments according to any of the preceding claims, wherein the GFP is EYFP further containing an F64L mutation, wherein X is 157, wherein the first protein of interest fused to the N-terminal fragment of GFP is fused to the C-terminal of the N-terminal fragment of GFP and wherein the second protein of interest fused to the C-terminal fragment of GFP is fused to the N-terminal of the C-terminal fragment of GFP.

26. The N-terminal fragment of GFP according to any of the preceding claims.

27. The C-terminal fragment of GFP according to any of the preceding claims.

28. Nucleic acid encoding a fragment according to any of the preceding claims.

29. A cell comprising an N-terminal fragment of GFP according to any of the preceding claims.

30. A cell comprising a C-terminal fragment of GFP according to any of the preceding claims.

31. A cell comprising the two GFP fragments according to any of the preceding claims.

32. A vector comprising the two GFP fragments according to any of the preceding claims.

33. A vector comprising the N-terminal fragment of GFP according to any of the preceding claims.

34. A vector comprising the C-terminal fragment of GFP according to any of the preceding claims.

35. A plasmid comprising the two GFP fragments according to any of the preceding claims.

36. A plasmid comprising the N-terminal fragment of GFP according to any of the preceding claims.

37. A plasmid comprising the C-terminal fragment of GFP according to any of the preceding claims.

38. A method for detecting the interaction between two proteins of interest comprising the steps of:

(a) providing at least one cell that contains two heterologous conjugates,
the first heterologous conjugate comprising a first protein of interest conjugated to an N-terminal fragment of GFP according to any of the preceding claims,
the second heterologous conjugate comprising a second protein of interest conjugated to a C-terminal fragment of GFP according to any of the preceding claims; and

(b) measuring the fluorescence from the at least one cell,

fluorescent cells indicating interaction between the two proteins of interest.

39. A method for monitoring the interaction between two proteins of interest comprising the steps of:

(a) providing at least one cell containing at least one stretch of nucleic acid encoding for two heterologous conjugates,

the first heterologous conjugate comprising a first protein of interest conjugated to an N-terminal fragment of GFP according to any of the preceding claims,

the second heterologous conjugate comprising a second protein of interest conjugated to a C-terminal fragment of GFP according to any of the preceding claims;

(b) culturing the at least one cell under conditions allowing expression; and

(c) measuring the fluorescence from the at least one cell,

fluorescent cells indicating interaction between the two proteins of interest.

40. A method according to any of the preceding claims, wherein the at least one cell is a mammalian cell.

FIGURE 1

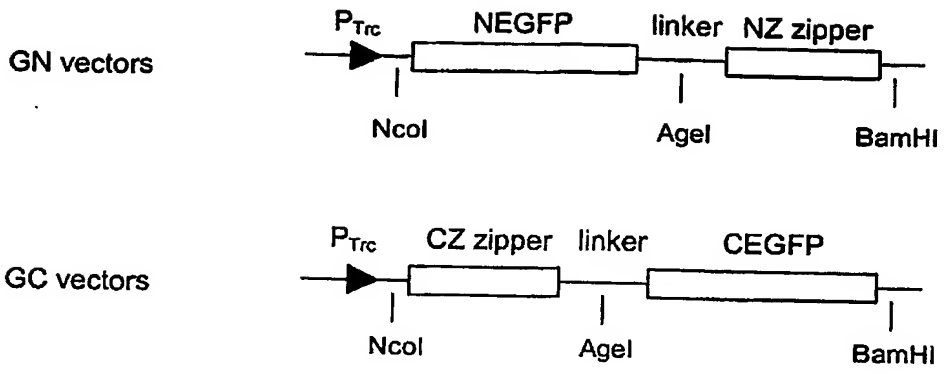


FIGURE 2

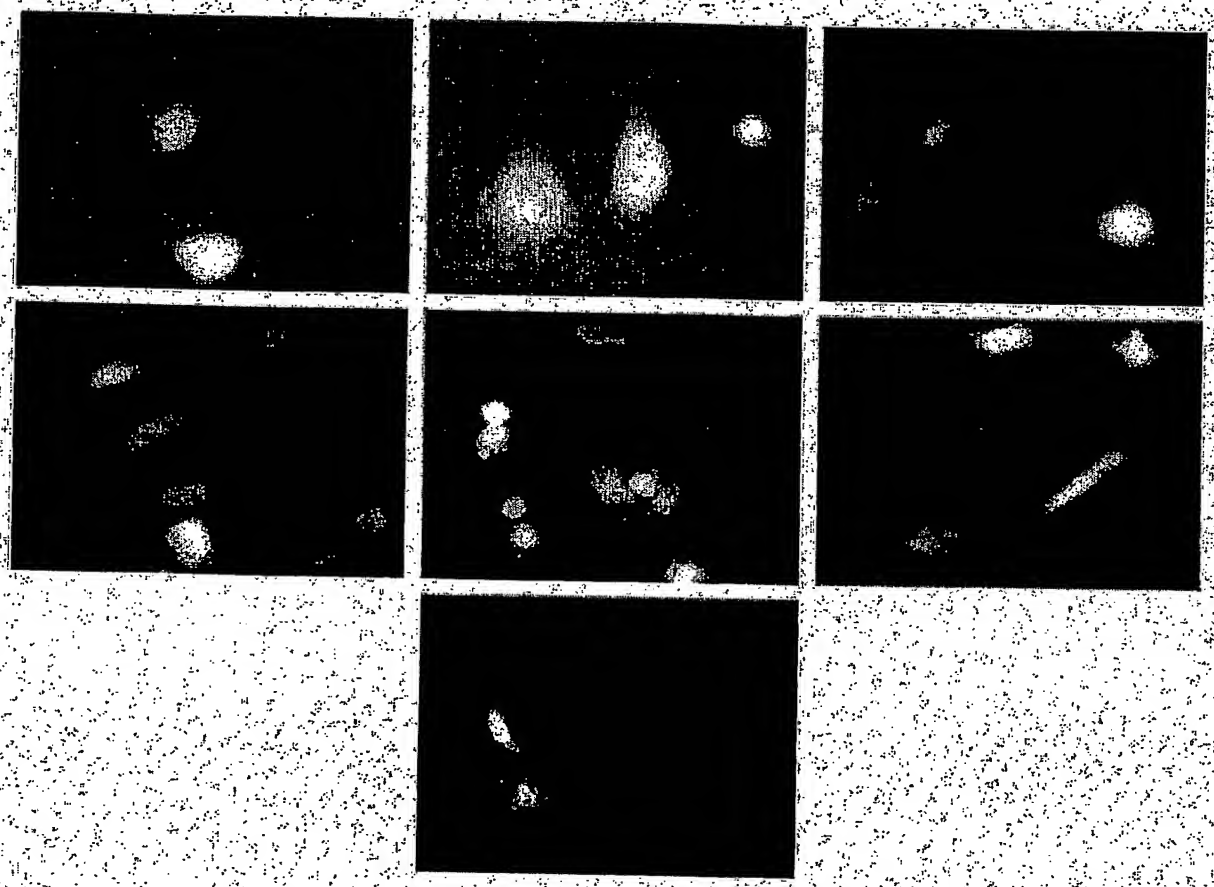


FIGURE 3

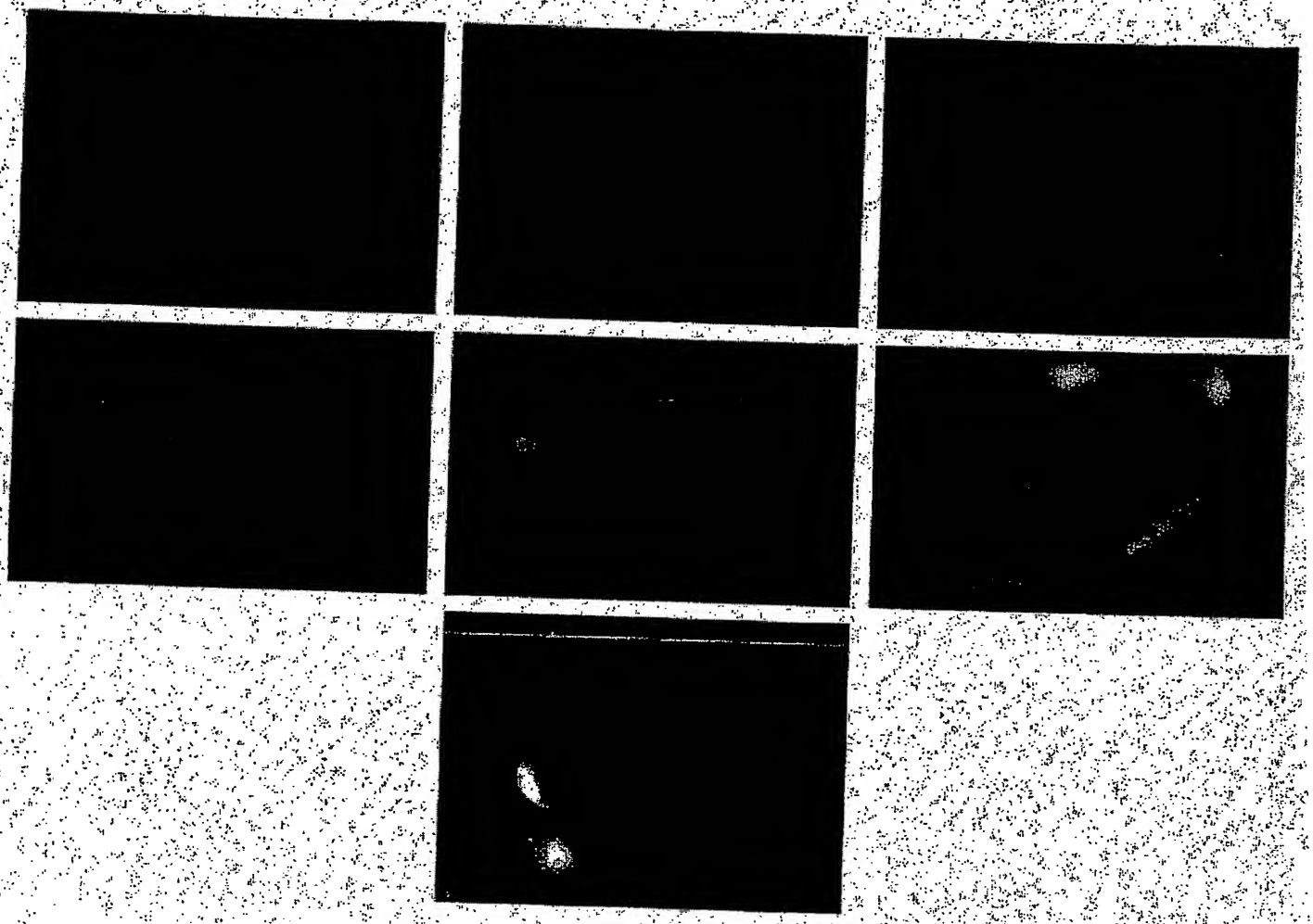


FIGURE 4

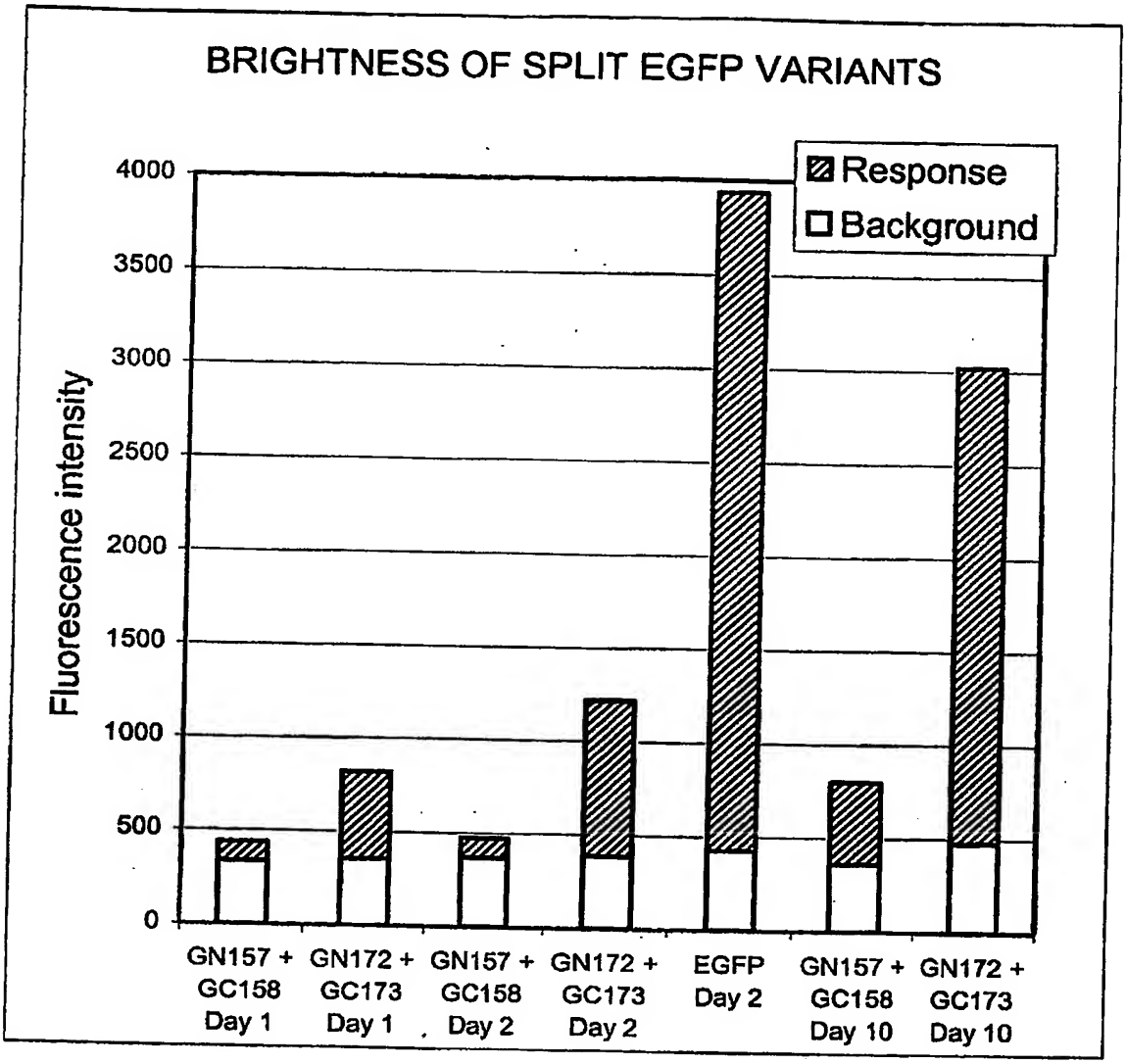


Figure 5

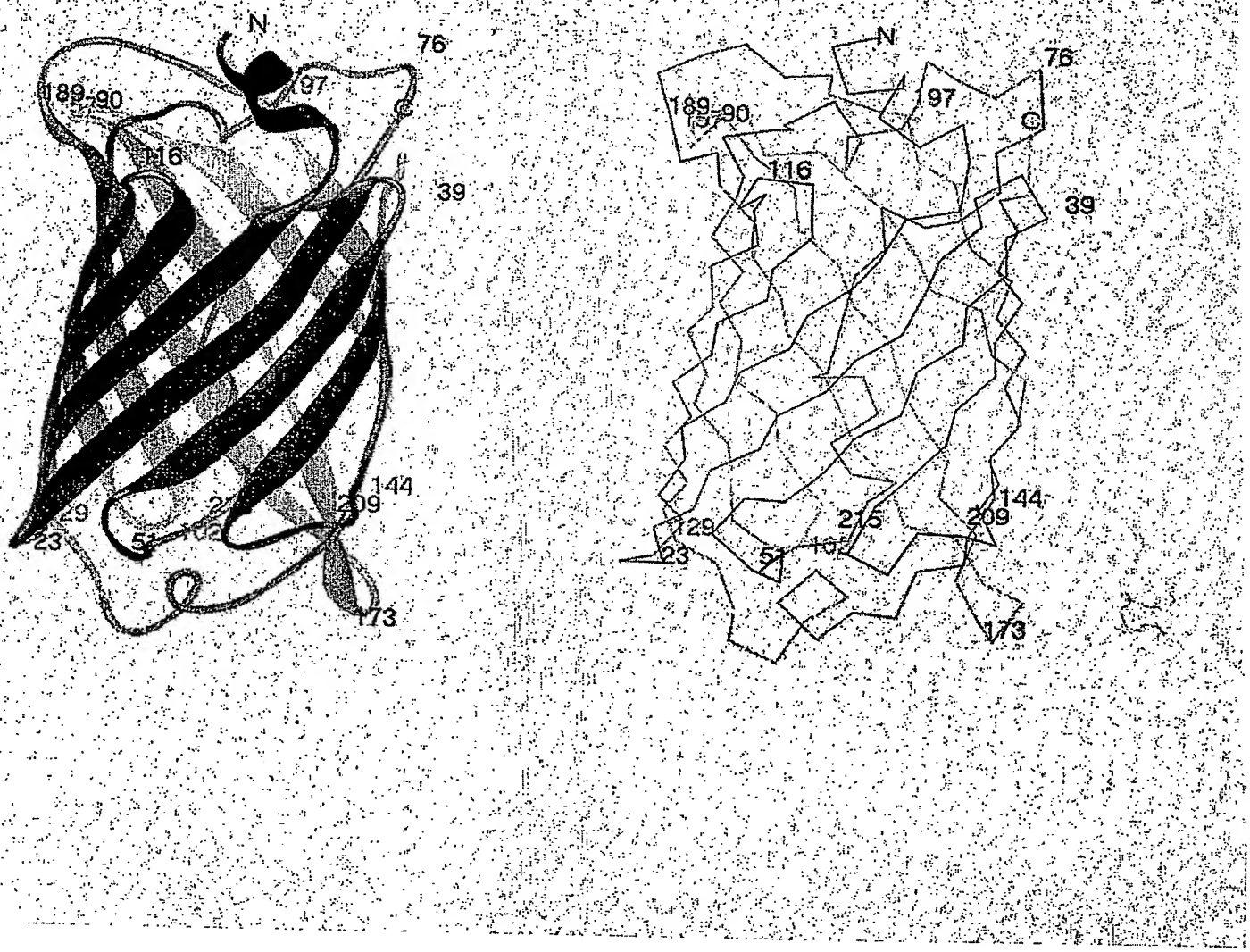


FIGURE 6

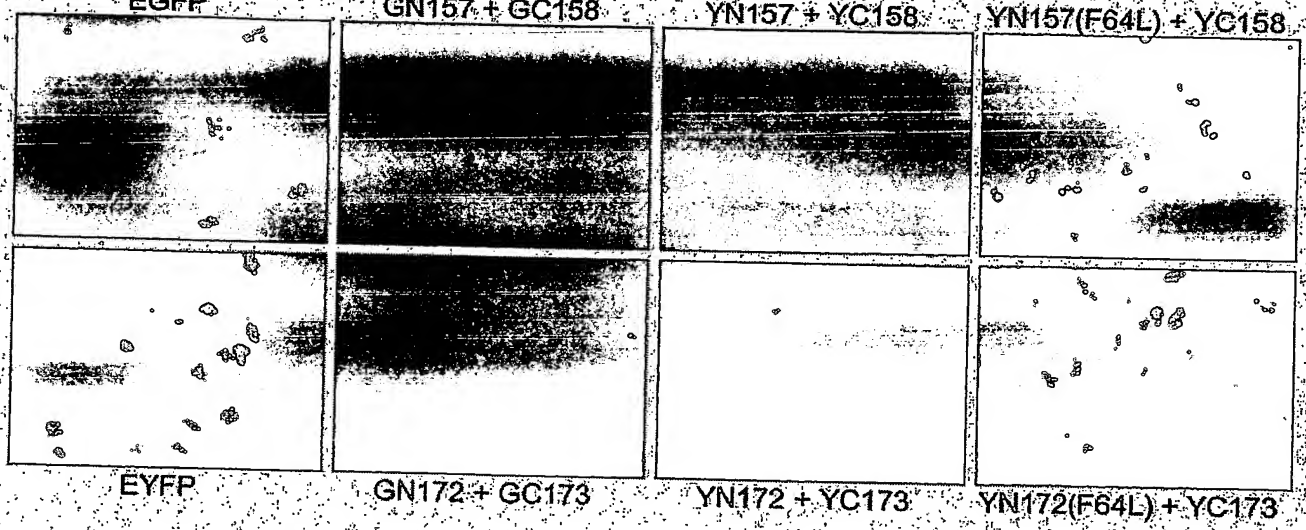


FIGURE 7

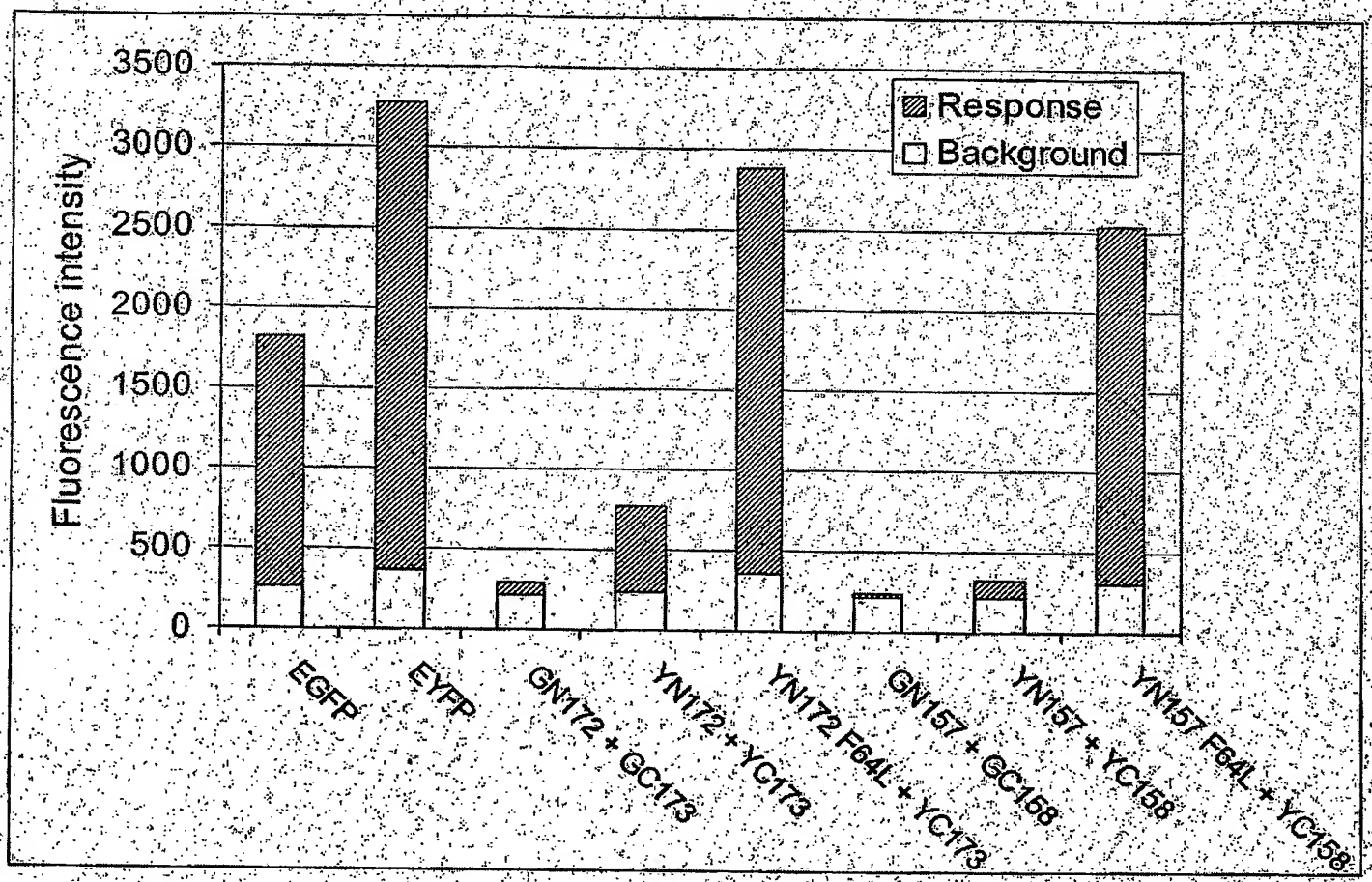


FIGURE 8

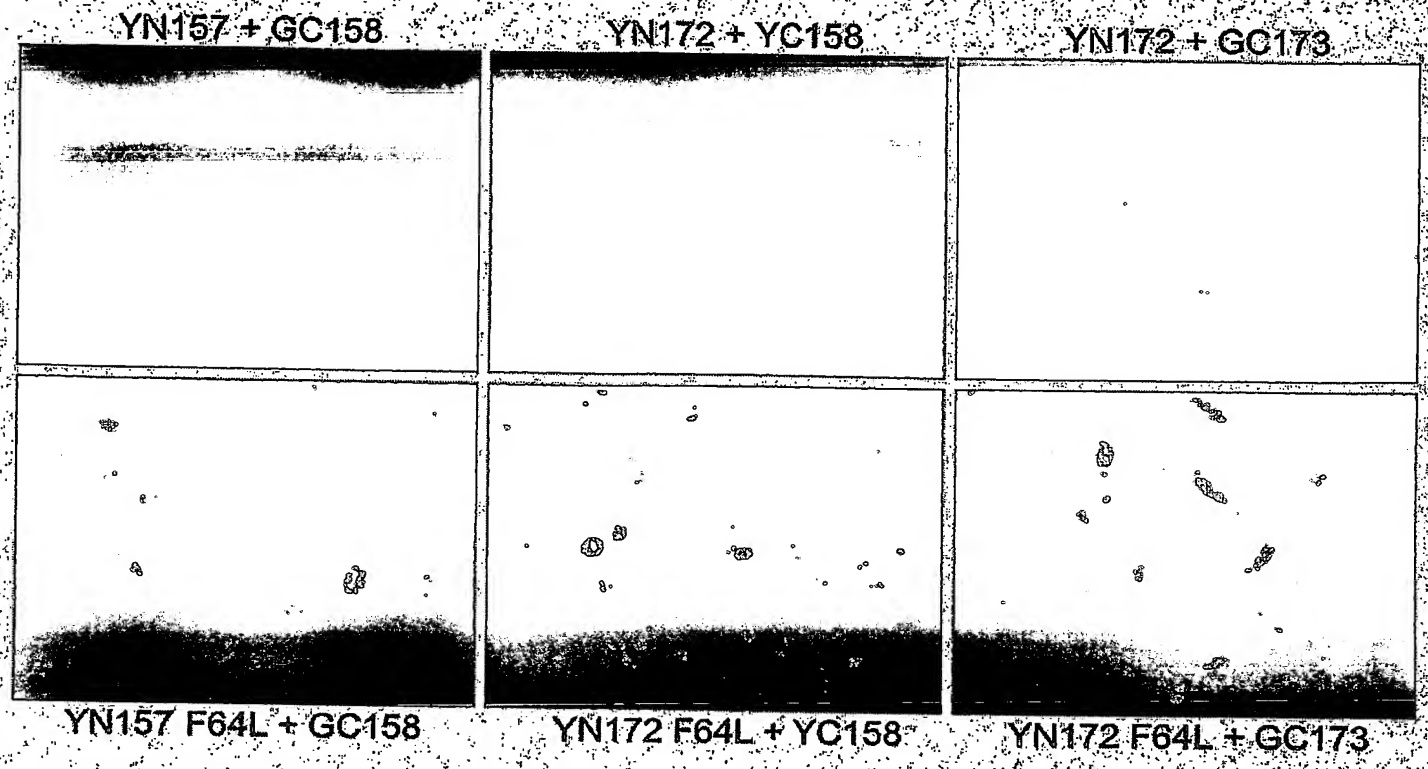
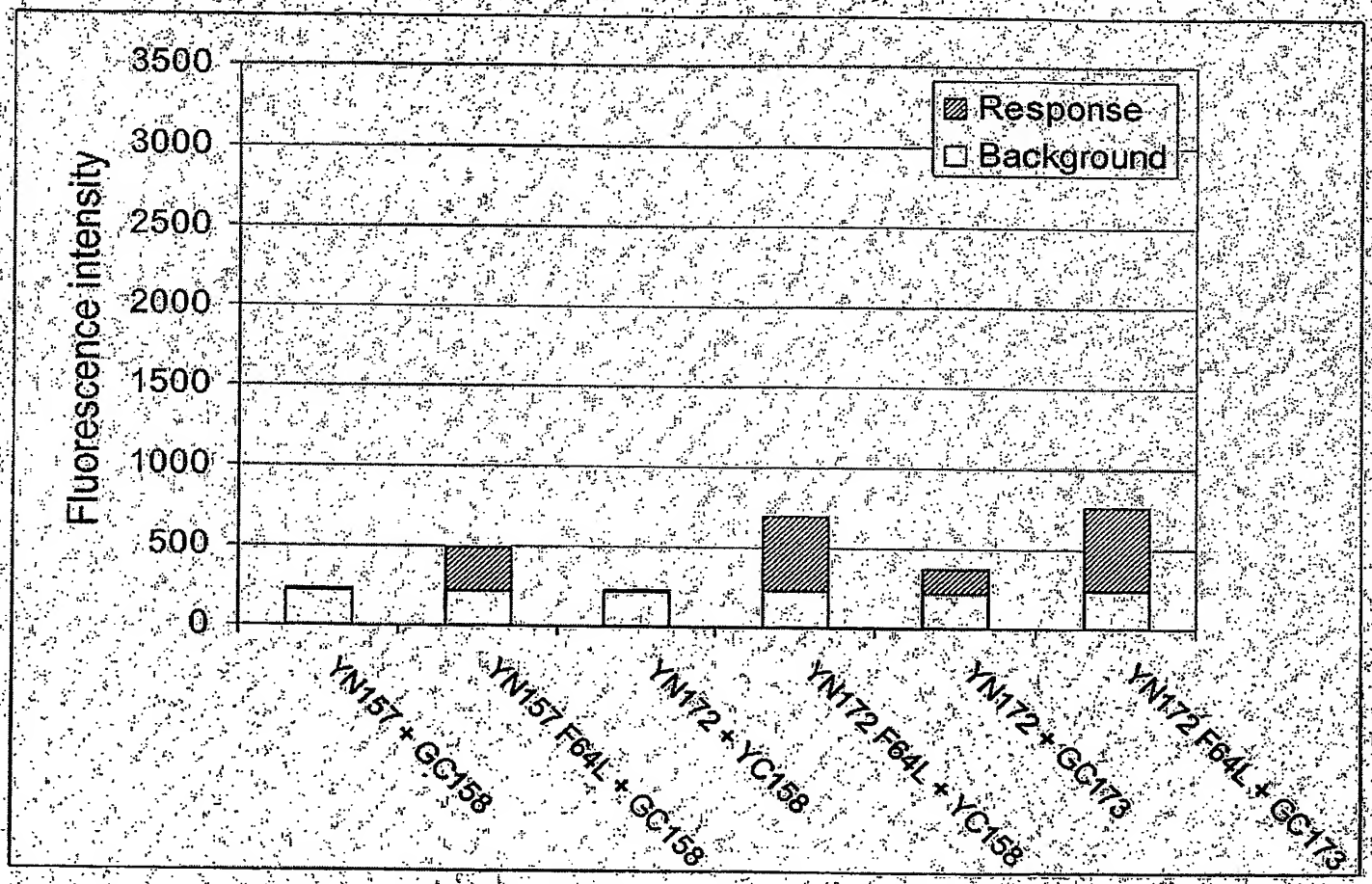


FIGURE 9



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